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SPORE GERMINATION OF FOMES SPECIES

FACULTY OF GRADUATE STUDIES AND RESEARCH

by



ICHIKO TSUNEDA

The undersigned certify that they have read, and recommend  
to the Faculty of Graduate Studies and Research, for acceptance,  
a thesis entitled "SPORE GERMINATION OF FOMES SPECIES" by  
ICHIKO TSUNEDA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
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IN

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DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "SPORE GERMINATION OF FOMES SPECIES" by ICHIKO TSUNEDA in partial fulfilment of the requirements for the degree of Master of Science in Mycology.





## ABSTRACT

Spore germinability of three species, *Fomes fomentarius*, *F. cajanderi*, and *F. igniarius* was examined on water agar and malt extract agar media. Spores of *F. fomentarius* germinated on malt extract agar, but not on water agar, while those of *F. cajanderi* and *F. igniarius* germinated on both media. *F. fomentarius* and *F. cajanderi* spores required about 8 hours to initiate germination, germinating in most cases within 24 hours. *F. igniarius* showed slow spore germination, requiring 3-5 days.

Viability of *F. fomentarius* spores was lost after 6 months and that of *F. cajanderi* spores was after 4-month storage period.

Maximum spore germination of *F. fomentarius* and *F. igniarius* was obtained at 22° C, and at 30° C, respectively. *F. cajanderi* spores germinated in a broad range of temperatures without a definite optimum temperature.

Spore germination of the three species was tested on the wood and bark of birch, aspen, balsam poplar and spruce. *F. fomentarius* spores germinated on all the wood with a slight preference for birch and aspen. In contrast, *F. cajanderi* showed spore germination to a similar extent on all the woods. Similarly, spore germination of *F. igniarius* was not particularly stimulated by any of the woods. All the species showed spore germination on spruce bark, but not on aspen and balsam poplar. A definite preference of these three *Fomes* species was not observed at spore germination stage.

During germination, *F. cajanderi* spores became swollen, while spores of *F. fomentarius* and *F. igniarius* did not. Germ tube emergence was



bipolar in *F. fomentarius* spores, but definite polarity was not observed with *F. cajanderi* and *F. igniarius*.

Ungerminated spores generally exhibited less differentiation in cell organelles than germinated spores. A striking difference was observed in cell wall among the species during germination. The cell wall thickness varied among the three species. The cell wall of ungerminated spores of *F. fomentarius* and *F. igniarius* had two layers and that of *F. cajanderi* had only one layer. The germ tube wall of *F. fomentarius* was continuous with the newly formed cell wall layer of the spore, while the germ tube wall of *F. cajanderi* was continuous with the original spore cell wall. No vacuoles were observed in ungerminated spores, but they were formed in germinated spores with electron-dense vacuole inclusions.





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## INTRODUCTION

The genus *Fomes* belongs to a large family of Homobasidiomycetes, the Polyporaceae which includes the greater proportion of wood-decay fungi. From the commercial point of view, these wood-decay fungi are most important agents since they destroy valuable raw material, and are the major cause of loss of timber and wood in service. Also, it should be pointed out that these fungi have an important role as decomposers in the turn-over of woody material in the forest ecosystem.

According to the accepted view, the natural distribution of these fungi is accomplished by spores and the common means of initiation of decay is through germination of spores of a suitable fungus after their deposition in contact with non-living wood that the fungus is capable of attacking and entering. Thus, in the life cycle of the wood-decay fungi, spore germination is the most critical stage.

In spite of the importance, there are very few studies on spore germination phase in the genus *Fomes*. The reason for this is undoubtedly attributable to a large extent to the difficulties that occur in the experimental conditions. Troublesome is the fact that the spores of these fungi are often difficult to collect or to store and often germinate very slowly at an extremely low percentage, if they can be induced to germinate. Moreover, the percentage of spore germination may vary considerably from one spore sample to another, because of contamination by other microorganisms, which frequently occurs in spore samples.

For this study, *F. fomentarius*, *F. igniarius*, and *F. cajanderi* were selected as experimental organisms, since they are commonly occurring species in this area, and the spores are usually discharged





from their sporocarps from early spring to late autumn. In addition, the spores are able to germinate relatively easily on artificial media.

Although some wood-decay fungi are quite specific as to substrate, in general, the majority are basically saprobic and are sufficiently adaptable, so that it is not unusual to find a species normally on conifers, occasionally occurring on hardwoods, or vice versa. Nevertheless, in spite of the lack of definite substrate-specificity, these fungi certainly show preference for specific kinds of woods. For example, in this area *F. fomentarius* is usually found on hardwoods, especially on birches and poplars. *F. igniarius* grows on a great variety of hardwoods, is not found on conifers (Boyce 1938), and shows a marked preference for aspen. On the other hand, *F. cajanderi* occurs commonly on dead conifers, and rarely on hardwood. Although the substrate-range of each wood-decay fungus is relatively well known (Boyce 1938; Overholts 1967) and the preferences of wood-decay fungi for certain kinds of wood are clearly demonstrated, virtually no explanation for these preferences has been given. It was assumed that the preference for certain kinds of wood could be related to spore germination requirements of each fungal species, since different species require different conditions for spore germination.

Another aspect of interest was the morphological and cytological changes during spore germination. A precise knowledge of a spore's characteristics is of cardinal importance in taxonomy. Surface morphology of spores in the Basidiomycetes is used as a taxonomic characteristic for many taxa. However, very little is known about this aspect of the genus *Fomes*, particularly at the ultrastructural level. Morphological and cytological information may be useful in distinguishing species of *Fomes*, since the generic limits of *Fomes* are not agreed upon



Bondarzew 1949; Overholts 1967). It was speculated that the mode of spore germination (the sequence of development of the spore during germination) as well as cytological features of the spores might be characteristic of each species of the genus *Fomes*. Thus, two aspects of the spore germination phase of these fungi were of particular interest in this study.

(1) Whether the spore germination requirements are different from species to species; (2) whether morphology and cytology of spore germination is characteristic of selected *Fomes* species. This thesis consists of five parts: (1) Spore sampling, (2) Spore germinability, (3) Spore longevity, (4) Factors affecting spore germination, and (5) Morphology and cytology of spore germination. The first three parts were conducted as preliminary studies.



## LITERATURE REVIEW

This literature review summarized the current state of knowledge concerning; (A) Factors affecting spore germination, and (B) Morphology and cytology of spore germination of wood-decay fungi. The first part deals with mainly the Polyporaceae, but the second part involves other groups of the Basidiomycetes, since too few studies have been done with the Polyporaceae.

### A. Factors affecting spore germination

Although basidiospore germination has been studied in many species of the Basidiomycetes, the conditions for spore germination have not been fully understood, particularly of wood-decay fungi. Spore germination will occur or not depending upon the number and relative importance of the favorable factors. The factors are: (1) moisture, (2) temperature, (3) gas, (4) pH, (5) radiation, (6) spore viability, (7) presence of other organisms, and (8) nutrients and substrates.

#### 1. Moisture

Comparatively little careful work has been done to determine the water requirements for spore germination of the Basidiomycetes. The spores of *Lenzites trabea* have been reported to show a high germination percentage when relative humidity is above 99% (Morton and French, 1966), although the exactness of the relative humidity assigned to various solutions may be questionable. Merrill (1970) suggested that spores of many heart-rotting fungi required free water to germinate, they sensitive to desiccation, and usually they did not survive long in the nature. In general, a higher germination percentage is obtained at a higher relative humidity.





## 2. Temperature

Temperature affects the time required for spore germination, the percentage of germination and the growth rate of germ tubes. The basidiospores of *Fomes pinicola* were found to germinate at temperatures ranging from 8 to 35° C, but the range of 22-30° C gave good germination (Mounce 1929). The spores of *F. igniarius* are reported to germinate well at temperatures in the range of 20-35° C (Good and Spanis 1958). Morton and French (1966) found that the highest percentage germination of *L. saepiararia* basidiospores occurred at temperatures from 29 to 35° C, and that maximum percentage germination of *L. trabea* basidiospores occurred at 25° and 40° C, although the reason for the bimodal curve in the case of *L. trabea* was not determined. The basidiospores of *Polyporus tomentosus* germinated well at 15-27° C with optimum germination from 20 to 24° C (Whitney 1966). Samajpati (1970) reported that the maximum stimulatory effect was 30° C for *F. lividus* basidiospores. Maximum germination (>75%) of *Pleurotus ostreatus* spores was reported to occur at 1° C (8 days) to 30° C (3 days), but to be poor below or above this range (McCracken 1974). Merrill (1970) indicated that the optimum temperature for spore germination of many heart-rotting fungi was within  $\pm 3^{\circ}$  C of the optimum for growth. Exposing spores to temperature above their maximum for germination for a prolonged period causes complete loss of viability. Such an example was reported by Scheld and Perry (1970) who found that the spores of *L. saepiararia* in free water lost viability in 1-3 days at 30° C. On the other hand, the results of exposure to low temperature are rarely lethal and under proper conditions spores can remain viable for years. *P. tomentosus* spores germinated after 20 months exposure to normal winter temperature,



at  $-18^{\circ}\text{C}$  in Saskatchewan (Whitney 1966). Also the basidiospores of *F. applanatus* (Brown and Merrill 1973) and *Pholiota annivella* (Lavallee and Lortie 1971) were reported to survive  $5^{\circ}$  and  $-25^{\circ}\text{C}$ , and  $-9^{\circ}\text{C}$  for 6 months, respectively.

### 3. Gas

Since most fungi are aerobic, it is not surprising that oxygen has been reported to be an absolute requirement for the germination of many fungus spores. Comparatively little is known about the effects of oxygen level upon spore germination of the Basidiomycetes, although Merrill (1970) assumed that oxygen was required for the germination of heart-rotting Hymenomycetes.

Morton and French (1974) reported that germination of *Polyporus dryophilus* basidiospores was stimulated by gaseous emanations of *Ceratocystis fagacearum*, and the stimulation appeared to be due to carbon dioxide, since its removal prevented germination, and the addition of  $\text{CO}_2$  stimulated germination. On the other hand, Brown and Merrill (1973) found that carbon dioxide did not induce the spore germination of *F. applanatus*. Thus, the effect of  $\text{CO}_2$  on spore germination is still open to question.

As to the effects of other volatile substances, McCracken (1974) reported that ethanol and ethylene apparently had little or no effect on germination of *P. ostreatus* basidiospores.

### 4. pH

Good and Spanis (1958) studied the factors affecting the germination of *F. igniarius*, and found that spores germinated best on 8% malt





extract medium buffered at pH 4.0, and none occurred on alkaline media. An examination of the influence of hydrogen-ion concentration on germination is also reported by Losel (1967), who suggested that the germination-stimulating activity of the acids tested was not a pH effect, but was due to direct entry of the acidic compounds into metabolic pathways. Samajpati (1970) reported that the maximum stimulatory effect on the spore germination of *F. lividus* was obtained at pH 5.5. Also, McCracken (1974) obtained optimum spore germination for *P. ostreatus* at pH 6, but found adverse effects of pH below 4.9 or above 6.9. Merrill (1970) reported that germination of fungus spores generally was favored by an acidic pH, and Cartwright and Findlay (1950) mentioned a similar response in connection with the Hymenomycetes. However, little is known about the effect of pH on spore germination.

## 5. Radiation

Mounce (1929) studied the effect of light on spore germination of *F. pinicola*, and stated that light retarded but did not inhibit germination. Samajpati (1970) indicated that alternate light and dark conditions gave the maximum stimulatory effect on the basidiospore germination of *F. lividus*. Different results were obtained with *P. ostreatus* spores by McCracken (1974). He found that the best germination was in darkness and was unaffected by 580-650 nm of light, but unfiltered or 390-530 nm of light caused a significant reduction.

## 6. Spore viability

The basidiospores of many species of Hymenomycetes are able to germinate as soon as they are liberated, and their viability seems to



last for quite a long time. Other species apparently require a latent period of several days between liberation and germination (Merrill 1970). Meyer (1936) stated that the germinating power of the *F. fomentarius* spore was of a rather long duration and that the spores showed 25% of germinating power after one year. Harrison (1942) examined the longevity of the spores of many Hymenomycetes, and listed the duration from 20 days for *P. ostreatus* to 175 days for *F. pinicola*. Similarly, Good and Spanis (1958) demonstrated that the spores of *F. igniarius* varied widely in their germinability, some spores germinated fairly well after 80 days, others failed to germinate after 10 days. Basidiospores of some Hymenomycetes seldom or never germinate under laboratory conditions, and even if they germinate, the amount of germination frequently is only a few percent, and often is less than 1% (Good and Spanis 1958; Hirt 1927; Miller 1962). White (1919) and Aoshima (1954) indicated that the low percentage of germination of *F. applanatus* was due to the low inherent viability of the spores or due to internal dormancy.

## 7. Presence of other organisms

It appears that spore germination of the Hymenomycetes is strongly affected by the presence of other microorganisms. Morton and French (1967, 1974) found that the spore germination of *P. dryophilus* was stimulated by 25% of the fungi tested, but *C. fagacearum* (Ascomycete) stimulated more germination than did other fungi. And they concluded that the stimulation appeared to be due to CO<sub>2</sub> emanation by these fungi. Similarly, Brown (1968), and Brown and Merrill (1973) reported that *F. applanatus* spores germinated well in the proximity of colonies of *C. fagacearum* as well as species of *Rhodotorula*, *Pullularia*, bacteria



and other fungi.

## 8. Nutrients and substrates

Many fungus spores germinate very poorly or not at all without added nutrients. Other fungi germinate fairly well in water, but do so better or more rapidly in nutrient media or in contact with complex biological products.

In an early study Mounce (1929) stated that the spore germination of *F. pinicola* did not occur in pine wood decoction. Good and Spanis (1958) found that extracts of aged wounds on aspen contained far less sugar and amino acids than extracts of fresh sapwood, but germination of *F. igniarius* var. *populinus* on the former was better. With the same organism, Wall and Kuntz (1964) examined the effect of cold-water extracts of trembling aspen on spore germination and found that cold-water extracts from dead branches markedly stimulated basidiospore germination. Later, Whitney (1966) indicated that autoclaved extracts from bark or wood stimulated basidiospore germination of *P. tomentosus*. Subsequently, Whitney (1971) obtained a high percentage of spore germination of the same fungus on media supplemented with bark extracts from roots of white and black spruce. Similarly, 80% germination of *F. pini* basidiospores was obtained on media containing cold water extracts of xylem from weeviled leaders dead 1 to 20 years and lateral branches dead 10 or more years (DeGroot 1965).

Price (1913) was the first to describe spore germination on wood. When he examined the spores of *Polyporus squamosus*, he observed the germination and penetration of wood or wood slips in pure culture. Meyer (1936) observed the germination of *F. fomentarius* on a water-soaked





block of wood. Basidiospores of *F. igniarius* var. *populinis* were reported to germinate when in contact with the outer sapwood of freshly cut stem sections of trembling aspen (Manion and French 1969). According to Paine (1968), the basidiospores of *Polyporus betulinus* germinated on non-sterilized microtome sections of non-host species - *Abies*, *Larix*, *Picea*, and *Tsuga*. Similar results were obtained using basidiospores of *F. pinicola*, and *F. subroseus*, which normally occur on conifers. Although the experimental method used is not clear, Lavallee and Lortie (1971) reported that the germination of *P. anrivella* basidiospores was better on solid than on liquid media, and that it was not influenced by the hosts (*Betula*, *Fagus*, *Acer*).

Good and Spanis (1958) examined some factors affecting spore germination of *F. igniarius* var. *populinus*, and suggested that stimulation by malt extract appeared to be due partly to the sugar content.

## B. Morphological changes of spores during germination

### 1. Light microscopic observations

In general, when spores are placed in a germination medium, swelling often occurs before germ tube formation, although there are only a small number of basidiospore studies. Rhoads (1918) mentioned that the spores of *Polyporus pargamensis* swelled up to less than twice their original size before putting forth germ tubes. Hirt (1927) examined the spore germination of *Polyporus gilvus* and noted that the first evidence of germination was a slight swelling, followed by the appearance of a germ tube. Similar evidence has been reported for *F. pinicola* by Mounce (1921). Recently, Nakai and Ushiyama (1974) have



reported that the basidiospores of *Lentinus edodes* became swollen after 2-hour incubation on potato sucrose agar.

When the basidiospores of *P. gilvus* germinated, they invariably put out a germ tube at the apex of the spore, that is, the end opposite the apiculus (Hirt 1927). According to this author, a single spore was never observed to put out more than one germ tube. It is interesting that the germination of *P. pargamensis* spores was found to occur generally, although by no means always, at the apiculate end of the spore. Almost always a single germ tube was put out at one end of the spore and very shortly after another was put out from the other end. Germination from the side of the spore was of rare occurrence (Rhoads 1918). Similar results were obtained with *F. applanatus* by White (1919). The spores of *F. applanatus* exhibited a definite polarity, the germ tube always issuing from the "truncated" apical end of the spore, and it almost equalled the spore in diameter. Mounce (1929) has reported that the swollen spores of *F. pinicola* might produce one to four germ tubes from the ends or sides of the oval spore. According to Nakai and Ushiyama (1974), the spores of *L. edodes* became swollen and then germinated by elongating in both directions along the long axis. Thus, there appear to be different modes in germ tube emergence among different species, particularly as to number of germ tubes and the position of the emerging germ tube.

Mounce (1929) reported that *F. pinicola* spores might become septate before the germ tubes were produced. On the other hand, Nakai and Ushinyama (1974) mentioned that the spores of *L. edodes* became septate as soon as they germinated and, after 18 hours, some of them were three - or four-celled. They also observed two vacuoles in an





elongated basidiospore.

## 2. Electron microscopic observations

By means of transmission electron microscopic studies, internal morphological changes of germinating spores can be demonstrated. Studies with replica techniques or scanning electron microscope reveal the surface features of spores. Although many studies had been made of fungal spores, no specific information had been available as to the fine structure of basidiospores until Voelz and Niederpruem (1964) reported on *Schizophyllum commune*. Since then, there have been several reports concerning both ungerminated and germinated basidiospores.

Voelz and Niederpruem (1964) described the basidiospore cell wall of *S. commune* as consisting of one fibrous layer approximately 73 nm wide. During germination a marked alteration in the form of the basidiospore occurred, and the cell wall of the germling was exceedingly difficult to fix by the fixative employed. Manocha (1965) reported that the basidiospore wall of *Agaricus campestris* showed three distinct layers. The outer layer was electron-opaque, smooth, and thicker than the two inner layers. The middle layer was less dense and appeared to have a fibrillar structure with numerous evenly dispersed granules. The inner layer was almost electron-transparent. Hyde and Walkinshaw (1966) found that *L. saepiaria* basidiospores possessed notably thin walls as compared with hyphal walls. The striking feature of the germinating spore was the very thin wall of the germ tube. A study of dormant and germinating spores of *Psilocybe* species has been made by Stocks and Hess (1970). The basidiospores of *Psilocybe* species had



a thick cell wall composed of three distinct layers. The outer layer was a fibrous layer and was continuous around the entire spore. It varied in texture from a thin hairy-like layer to a covering of long, loose fibers. The middle layer was a thick, electron-dense layer of constant thickness. The inner layer was more electron-transparent than the middle layer and was about one quarter as thick. The thickness of the inner layer was constant, except apiculus areas. As germination continued the outer two layers of the cell wall appeared to break down and allow the passage of the germ tube. The inner layer of the wall appeared to be both stretched and dissolved during germination. Later in germination the protoplast penetrated the three layers of the cell wall and the germ tube developed into a small bubble outside of the spore. Heintz and Niederpruem (1971) carefully examined the germinating basidiospores of *Coprinus lagopus*. The basidiospore cell wall was composed of 6-layers and had a germ pore. At the onset of germination, the wall of the germ tube of basidiospores of *C. lagopus* was formed from the innermost spore wall layer. Using shadowed sections, Griffiths (1971) observed that the basidiospores of *Panaeolus campanulatus* possessed a 2-layered wall, an outer layer which was electron-dense and faintly fibrillar in structure and an inner layer, less electron-dense and composed of loosely arranged fibrils. At the germ pore, the outer layer was reduced to a thin covering and the inner cell wall was absent. Similarly, Nakai and Ushiyama (1974) found that the basidiospore cell wall of *L. edodes* consisted of the two layers, i.e., the electron-dense inner layer and more or less electron-lucent outer layer. With development of swelling and elongation of an incubated spore, the inner layer of a spore wall increased in thickness, but the



outer layer appeared to be unchanged at all the stages of germination.

Although cell membranes are major structural and functional components of cells, those of basidiospores have not been carefully examined. Voelz and Niederpruem (1964) reported that the cell membrane of *S. commune* basidiospore measured 6 to 8 nm. The cell membranes of *L. saepiaria* and *C. lagopus* basidiospores have been reported to show invagination at irregular intervals by Hyde and Walkinshaw (1966), and Heintz and Niederpruem (1971), respectively. Stocks and Hess (1970) reported an interesting observation of *Psilocybe* species basidiospores. The cell membrane normally continued numerous invaginations, and was closely adpressed to the inner cell wall, but in germinating spores it often pulled away from the cell wall. Freeze-etching technique provided more information of membrane surface. Griffiths (1971) showed that the cell membrane bore numerous randomly-arranged particles, and was frequently invaginated. The invaginations showed no apparent pattern, varied in length from 0.2-0.5  $\mu\text{m}$  and were generally 30 nm in width.

The number of nuclei in basidiospores seems to vary according to species. The basidiospores of *S. commune* (Voelz and Niederpruem 1964; Aitken and Niederpruem 1970), *Psilocybe* species (Stocks and Hess 1970), and *C. lagopus* (Heintz and Niederpruem 1971) are reported to be binucleate. Those of *L. saepiaria* (Hyde and Walkinshaw 1966) and *P. campanulatus* (Griffiths 1971) appear to be either uninucleate or binucleate. Nakai and Ushiyama (1974) have reported that the *L. edodes* basidiospores were uninucleate, and that the nucleus elongated and divided into two in germinating spores. In general, nuclear behavior during the germination process is not clear in many basidiospores.







Voelz and Niederpruem (1964) reported that the mitochondria of fresh basidiospores of *S. commune* were poorly defined, and appeared as loosely assorted membranes or vesicles even if they were fixed in  $\text{KMnO}_4$ . But during germination the mitochondria appeared to be more organized. However, more information is required, since there are few studies of mitochondria of basidiospores, especially as to the changes in the size, shape, and number of mitochondria during spore germination.

Aitken and Niederpruem (1970) noted that endoplasmic reticulum (ER) was sparse or absent in ungerminated *S. commune* basidiospores. Similarly, Voelz and Niederpruem (1964), Hyde and Walkinshaw (1966), Stocks and Hess (1970) and Griffiths (1971) did not observe ER in both dormant and germinated spores. On the other hand, Aitken and Niederpruem (1970), Heintz and Niederpruem (1971) and Nakai and Ushiyama (1974) recognized more frequent presence of ER in germinated spores of *S. commune*, *C. lagopus*, and *L. edodes*, respectively. In general, many fungi studied showed paucity of ER in ungerminated spores. Thus, Sussman and Halvorson (1966) concluded that one of the most important ultrastructural events associated with the germination of fungus spore was the formation of ER. However, Wells (1964) and Manocha (1965) observed ER in ungerminated basidiospores of *Exidia nucleata* and *A. campestris* respectively. It is still too early to draw any conclusions because of the limited number of studies.

It is known that some fungal spores contain stored food in the form of lipid, while others contain glycogen. Lipid droplets are formed predominantly in ungerminated basidiospores (Hyde and Walkinshaw 1966; Stocks and Hess 1970; Aitkens and Niederpruem 1970; Griffiths 1971; Wells 1965; Manocha 1965; Nakai and Ushiyama 1974). From the results of



histochemical experiments together with electron microscopic studies, Voelz and Niederpruem (1964) suggested that the dormant spores of *S. commune* might possess carbohydrates rather than lipids, and in the germinating process the lipid droplets might appear. In both ungerminated and germinated basidiospores of *C. lagopus*, lipid droplets as well as glycogen appeared to be present (Heintz and Niederpruem 1971).

Whether vacuoles are present or not in ungerminated basidiospores is not clear. Griffiths (1971) suggested the probable presence of vacuoles in the dormant spores of *P. campanulatus*. However, it has been reported that vacuolization takes place during germination process (Voelz and Niederpruem 1964; Hyde and Walkinshaw 1966; Stocks and Hess 1970; Aitken and Niederpruem 1970; Heintz and Niederpruem 1971; Nakai and Ushiyama 1974). Hyde and Walkinshaw (1966) suggested that these inclusions (osmiophilic bodies) observed in germinated spores were possibly formed by coalescence of the smaller lipid bodies, which appear to be fewer in number in germinated spores. The fact that the inclusions were always associated with a large vacuole and vesicular membrane system indicates that this entire complex may represent the mobilization of nutrients for the emerging germ tube. A second possibility suggested is that the vacuole in the spore may be formed by loss of cytoplasmic constituents into the germ tube.

Numerous ribosomes appeared to be common in *L. saepiaria* (Hyde and Walkinshaw 1966) and in *Psilocybe* species (Stocks and Hess 1970) basidiospores. Hyde and Walkinshaw (1966) also observed polysomes and



vesicular bodies.

Golgi has been reported only by Nakai and Ushiyama (1974) in *L. edodes* basidiospores.

Lomasomes were not found in the basidiospores so far studied.





## MATERIALS AND METHODS

### A. Spore sampling

#### 1. *Fomes fomentarius* and *F. igniarius*

All the spores used for the study on germinability, longevity, and the factors affecting spore germination were obtained from sporocarps on dead birch trees found in Emily-Murphy Park, Edmonton. The spores used for cytological study were collected in either Emily-Murphy Park, or The Devonian Botanic Garden, Devon, from April to August, 1976. Spores were collected on sterile glass slides suspended 1-2 cm below sporulating sporocarps by masking tape, so that the spores fell directly upon the glass surface (Fig. 1). The density of spores on the glass slides varied widely with the weather, the season, and the sporocarps. All the slides with a dense spore deposit were then wrapped in a clean aluminum foil, on which the date of collection and sporocarp number were labelled. Trees used in this study were designated by a number, and each sporocarp on a tree was assigned a number. Wrapped slides were kept at room temperature until the spores were used. Most of the spores were used immediately after collection.

#### 2. *F. cajanderi*

The spores of this species were obtained from the sporocarps on a spruce in The Devonian Botanic Garden. These collections of the spores often were associated with other microorganisms which usually interfered with the observation of spore germination of *F. cajanderi*. However, *F. cajanderi* often forms sporocarps in culture from which spores are



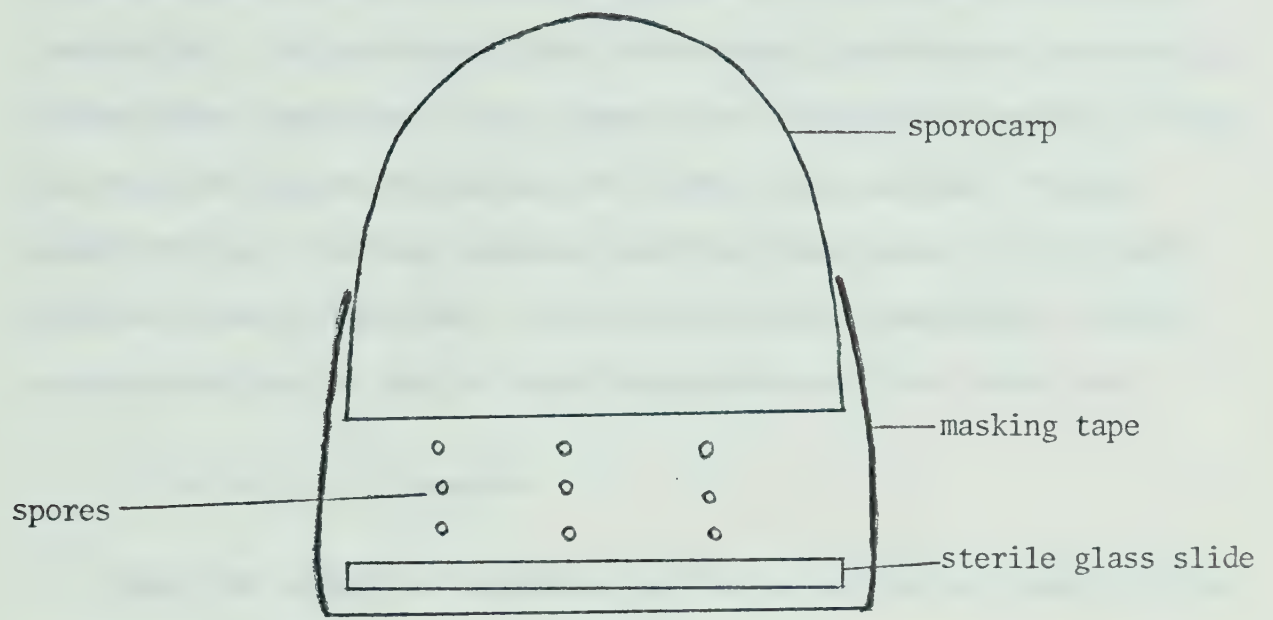


Fig. 1. Method of spore collection



obtained. The spores formed in culture are indistinguishable from those obtained from natural sporocarps (Wong 1973). Therefore, spores were also collected from cultures of isolate B19 and used. Petri dishes of 2% glucose and 2% malt extract agar (Appendix 1) were inoculated with isolate B19. The inoculated petri dishes were placed near a south-facing window under conditions of room temperature and relative humidity. After the mycelium covered the medium, the dishes were inverted. Within 5 weeks fruiting structures appeared and from these spores of relatively uniform age were collected. The spores were used immediately after the spore deposition, or kept at room temperature until they were used.

### 3. Variability of samples

Since the effects of different factors on germination could only be determined when comparable samples of spores were available, several groups of samples were tested to determine the extent of their inherent variability. The following groups of samples were tested: (1) Spores collected simultaneously from sporocarps on different trees; (2) Spores collected simultaneously from different sporocarps on the same tree; (3) Spores collected from the same sporocarps on different dates. The information from these preliminary tests (Appendix 2) indicated that differences between spore samples existed, that they might be of considerable magnitude, and that appropriate precautions must be taken. Accordingly, materials for each comparative study were obtained from several sporocarps on the same date, and the spores were mixed in order to obtain sufficient quantity for each germination study.





## B. Germinability of spores

Spores of each species were collected in a sterile petri dish by adding sterile distilled water and scraping the glass slide or petri dish lid surface with an sterile inoculation loop. The spore density was adjusted to the range of:  $1.1 - 3.1 \times 10^7$  spores/ml for *F. fomentarius*;  $0.8 - 2.2 \times 10^6$  spores/ml for *F. cajanderi*;  $2.4 - 6 \times 10^6$ /ml for *F. igniarius*. One or two drops of each spore suspension were inoculated with a sterile pasteur pipette onto 2% malt extract agar and 1% water agar media (Appendix 1) in petri dishes. The spores were spread evenly over the agar surface by a sterile inoculation loop and allowed to germinate at room temperature (22°C) under diffuse light. Observations on the number of germinated spores were made after 0, 4, 8, 12, and 24-hour incubation for *F. fomentarius* and *F. cajanderi*, and after 0, 3, 5, and 7-day incubation for *F. igniarius*. For each observation 100 spores were counted in 10 fields scattered at random over a petri dish or, if contamination had occurred, in regions free from contamination. Three replicas were made, and the same experiment was repeated three times for each species. Although the possibility that the density of spores on the agar media might affect germination was considered, little variability in percentage germination was observed in the range of spore density used. For the assessment of germination, the spores were defined as germinated when the germ tube was as long as it was broad.



### C. Longevity of spores

To determine the extent to which spores of *Fomes* species were viable, the longevity of spores was studied. Since spores collected from different sporocarps, or on different dates varied widely in their germinability, only one collection with sufficient quantity of spores was tested for each species. The spore collection of *F. fomentarius* was collected on a sterile glass slide on April 28th, and that of *F. cajanderi* was on a petri dish lid on November 15th, 1976. The spores were kept at room temperature, and after various periods, the spores were removed from the dry glass slide or petri dish lid by lightly touching with a loop of sterile water. Then the spores were inoculated on 2% malt extract agar, and percentage germination was examined after 24-hour incubation at room temperature.



## D. Factors affecting spore germination

### 1. Effect of temperature on spore germination

Spore preparation was the same as that described in section B (Germinability of spores). Spores were inoculated on 2% malt extract agar and 1% water agar media. They were incubated at 15, 20, 22, 25, and 30°C for *F. fomentarius* and *F. igniarius*, and 10, 15, 20, 22, 25, 30, and 35°C for *F. cajanderi*. The petri dishes were kept in sealed polyethylene bags and placed in the dark. Germination percentage was counted after 24-hour incubation for *F. fomentarius* and *F. cajanderi*, and after 5-day incubation for *F. igniarius*, since the spores of the latter species germinated slowly under these conditions. Observations were made in the same manner as described in section B. Three replicas were made for each species and the experiment was repeated three times.

### 2. Effect of substrate on spore germination

Aspen (*Populus tremuloides* Michx.), Balsam Poplar (*P. balsamifera* L.), Birch (*Betula papyrifera* Marsh.), and Spruce (*Picea glauca* (Moench) Voss) trees without decay were cut in October, 1975, at The Devonian Botanic Garden. The logs were brought into the laboratory and the bark (outside the cambium) was separated from the sapwood by scraping with a large knife. The bark was cut into approximately 2.5 x 2.5 cm squares, and wood blocks approximately 2.5 x 2.5 x 0.8 cm in size were made. Both bark pieces and wood blocks were placed separately in sealed plastic bags, and stored at -20°C until used.

Dialysis membranes, 1.5 x 1.0 cm in size, which had been immersed in boiling distilled water for a few hours, were sterilized in distilled





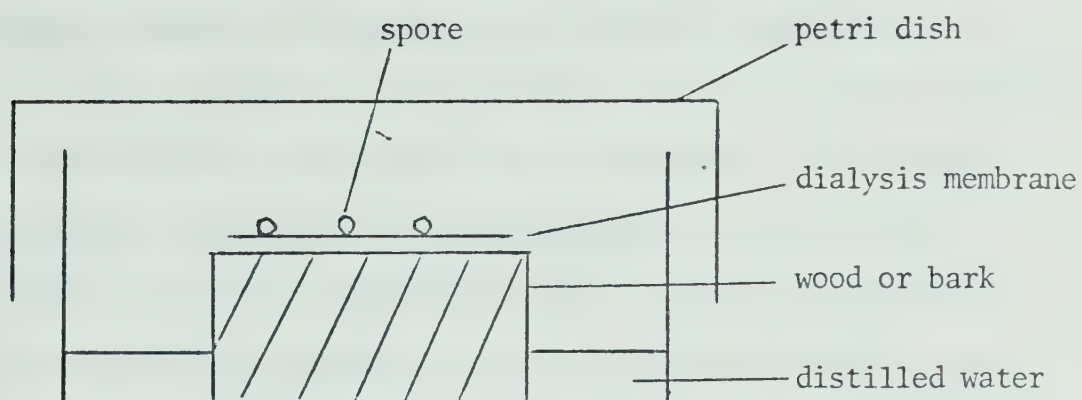


Fig. 2. A model of membrane method for studying germination on wood or bark.

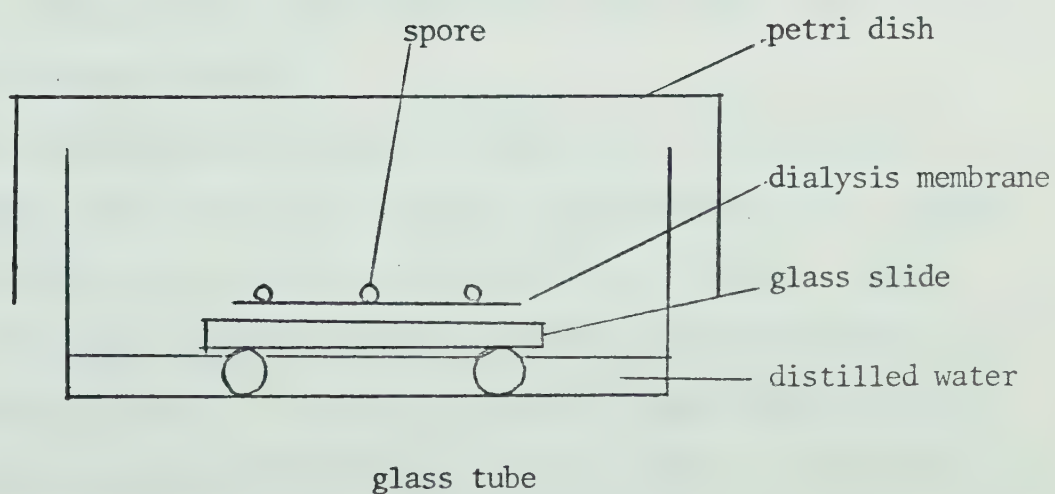


Fig. 3. A model of membrane method for studying germination on distilled water.



water at 121°C for 20 minutes at 15 lb. pressure.

A dialysis membrane was placed on the surface of each defrosted wood or bark sample, which had been partially immersed in sterile distilled water in a sterile petri dish to keep the tissues moist. The membrane was held in contact with the wood or bark surface by the free water present between the membrane and tissue (Fig. 2). Since the inside of petri dish was moist, the membrane did not dry up during investigation.

On the membrane, one or two drops of spore suspension were applied by using a sterile pasteur pipette. The spore densities were  $4.3 - 30 \times 10^6$  spores/ml for *F. cajanderi*, and  $2.4 - 4.4 \times 10^5$  spores/ml for *F. igniarius*. When the spore suspension was dropped from 3 cm above the membrane surface, the spores spread evenly on the membrane, so that there was no need to spread the spores with needles.

For controls, the membranes were placed on a sterile glass slide kept in a sterile petri dish with 5 ml of sterile distilled water (Fig. 3). Also, 2% malt extract agar and 1% water agar media in petri dish were used for controls.

After the incubation period of 24 hours for *F. fomentarius* and *F. cajanderi*, and 5 days for *F. igniarius*, the membranes were removed from the wood, bark, or glass slide surfaces, and placed on a glass slide. The percentage germination was counted in the same manner as described in section B. The counting had to be done quickly under a light microscope, since the membranes dried up easily on the glass slide. Three replicas for each wood or bark were made and the experiment was repeated three to seven times.



## E. Morphology and cytology of spore germination

### 1. Morphology

#### 1) Light microscopy

The spore suspensions of *F. fomentarius* and *F. cajanderi* with the spore density about  $3.0 \times 10^7$  spores/ml and  $2.0 \times 10^6$  spores/ml, respectively, were dropped on dialysis membranes (1.5 x 1.0 cm) that had been placed on 2% malt extract agar in petri dishes. After 24-hour incubation at room temperature, the membranes were removed from the agar medium, and placed in a petri dish. The spores on the membranes were stained with acridine orange (Appendix 3). Although this acridine orange stain is used for fluorescence microscopy, the observations were made under phase contrast and Nomarski-interference.

#### 2) Scanning electron microscopy

One or two drops of spore suspension with the same spore density as described for light microscopy were smeared over 2% malt extract agar. Drops were applied directly over wood blocks of birch and spruce, which had been prepared in the similar manner to that described in section D, except that the wood blocks were about 0.5 x 0.5 x 0.3 cm in size. After 18-hour incubation, agar blocks (0.5 x 0.5 cm) were cut from the agar medium in the petri dish. These agar blocks and the wood blocks were transferred to a petri dish (4 cm diameter) to which 3% glutaraldehyde in 1/10 M  $\text{Na}_2\text{HPO}_4$  -  $\text{KH}_2\text{PO}_4$  buffer (pH 7.0) was added to immerse the blocks. The spores on the blocks were fixed overnight at room temperature, washed with distilled water twice, 15 minutes each time, and post-fixed





in 2% Os O<sub>4</sub> in the same buffer for 3-4 hours. The fixed materials were rinsed three times with distilled water, frozen quickly in Freon 22, stored briefly in liquid nitrogen, and freeze-dried in vacuum at ~ - 70°C (Nei, 1974). The agar and wood blocks with freeze-dried spores were stuck to stubs with conductive glue and coated with carbon and gold in a vacuum evaporator. In addition to the freeze-drying method, critical-point drying method was also used. The material, fixed as described for freeze-drying, was dehydrated in ethanol-water series (Appendix 4) and taken through the absolute ethanol-amyl acetate series (Appendix 5) into amyl acetate. The material was critical-point dried in a Denton Vacuum DCP Unit using carbon dioxide (Anderson 1951), and shadowed twice with gold. Micrographs were taken in a Cambridge Stereoscan S<sub>4</sub> scanning electron microscope.

## 2. Cytology

### 1) Transmission electron microscopy

For transmission electron microscopy, ungerminated spores of *F. fomentarius*, *F. igniarius* and *F. cajanderi* and germinated spores of *F. fomentarius* and *F. cajanderi* were examined. Since the spores of *F. igniarius* germinated slowly, and the spore collections were contaminated more often than other species, a sufficient quantity of germinated spores of *F. igniarius* could not be obtained. Since a large amount of spore material is required for transmission electron microscopy, a thick layer of spores collected on sterile glass slides was removed with a sterile inoculation loop, suspended in 3% glutaraldehyde in 1/10 M Na<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0), and fixed overnight in the



glutaraldehyde at room temperature. The spores were centrifuged and washed with the same buffer for 15 minutes twice. The washed spores were pelleted by centrifugation and embedded in a drop of 1% water agar. The agar-embedded materials were cut into small pieces, fixed in 2%  $\text{OsO}_4$  in the same buffer for 4 hours at room temperature, dehydrated in an ethanol series, taken to propylene oxide (Appendix 6), and embedded in Araldite 502 (Appendix 7). In the preparation of germinated spores, a part of spores on the slides was suspended in a small amount of sterile distilled water. The spore suspension was transferred into flasks containing 50 ml of 2% malt extract in distilled water. These flasks were placed in a shaker at room temperature for 18 hours. Then the germinated spores were centrifuged, washed twice with distilled water, and embedded in 1% water agar. After fixation and dehydration, Araldite blocks were prepared as described previously. From the Araldite blocks, gold and silver sections were cut on a Sorvall Porter-Blum ultra-microtome MT-2 with glass knives. The thin sections were stained with alcoholic 2% uranyl acetate for 5 minutes, followed by aqueous 0.2% lead citrate for 2 minutes. Electron micrographs were taken in the Philips EM 200 and 201.

## 2) Light microscopy with histochemical techniques

The relatively large spores of *F. fomentarius* were examined with several histochemical techniques using Feder and O'Brien's methods (1968). Methods for the preparation of plastic blocks were the same as those used in the study of transmission electron microscopy, with the following modifications. The germinated spores were fixed in 3% glutaraldehyde in



1/15 M  $\text{Na}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer (pH 6.8) at 4°C. Agar embedded spores were directly dehydrated in an ethanol series, taken to propylene oxide, and embedded in Spurr's standard medium (Appendix 8) and Epon 812 (Appendix 9). A part of the glutaraldehyde-fixed spores was post-fixed with 2%  $\text{OsO}_4$  in 1/15 M  $\text{Na}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer (pH 6.8) for 4 hours. The fixed materials were dehydrated and embedded in Araldite 502 (Appendix 7). The ungerminated spores were fixed with the same procedure and embedded in Araldite. Plastic blocks were sectioned on a Reichart Om U2 ultramicrotome with glass knives. Sections 1.0 - 1.5  $\mu$  in thickness were mounted on gelatin coated glass slides (Jensen 1962). The sections were directly examined or stained with Toluidine Blue O, Periodic acid-Schiff, IKI, and Sudan Black B (Appendix 10).





## RESULTS

### A. Germinability of spores

Spore germination of three species, *Fomes fomentarius*, *F. cajanderi*, and *F. igniarius* was observed on 2% malt extract agar and 1% water agar. The percentage germination of each species is given in Tables 1-3.

Spores of *F. fomentarius* germinated on 2% malt extract agar, but not on 1% water agar. The percentage germination at 8-hour incubation was low (5%), but it increased rapidly and reached 67% after 24 hours. After this period, an exact germination rate could not be obtained because of the profuse mycelial growth on the medium.

Spores of *F. cajanderi* germinated on both media, showing similar germination rates. They began to germinate after 8 hours and the percentage germination reached 22-24% at 24-hour incubation. As with *F. fomentarius*, the mycelial growth of *F. cajanderi* was very rapid after spore germination and covered the medium after 24 hours. Therefore, the germination rate could not be counted after this period.

*F. igniarius* exhibited slow spore germination, with a very low percentage germination on both media. The germination rate was slightly higher on 2% malt extract agar than on 1% water agar. It was only 7% on 2% malt extract agar at 3-day incubation and after 7 days the percentage germination reached only 14%. After this period serious contamination by other microorganisms took place on both media, which interfered counting germinated spores.

### B. Longevity of spores

The viability of spores of *F. fomentarius* and *F. cajanderi* was



Table 1. Percentage germination of *F. fomentarius* spores on agar media at different incubation periods.

Media	0	4	8	12	24 hr
2% malt extract agar	0	0	5	21	67%
1% water agar	0	0	0	0	0

Table 2. Percentage germination of *F. cajanderi* spores on agar media at different incubation periods.

Media	0	4	8	12	24 hr
2% malt extract agar	0	0	0	16	24%
1% water agar	0	0	(+) <sub>a</sub>	15	22
			0		
			(+) <sub>a</sub>		

a: Less than 1% spore germination.

Table 3. Percentage germination of *F. igniarius* spores on agar media at different incubation periods.

Media	0	3	5	7	
2% malt extract agar	0	7	14	14	%
1% water agar	0	0	9	12	



examined periodically, and the results on longevity of the spores are shown in Tables 4 and 5.

Some of the spores collected on dry glass slides shriveled noticeably in a short time, while others remained turgid even after prolonged storage. In general, shriveled spores failed to germinate and turgid spores remained viable for a considerable period.

Spores of *F. fomentarius* did not show any loss of viability after 1-month storage, giving 76% germination. However, there was a gradual decrease in viability with 55% germination at the 3-month storage period, and complete loss of viability was observed after 6 months.

The viability of spores of *F. cajanderi* decreased gradually with storage period. *F. cajanderi* lost its spore viability earlier than *F. fomentarius*. The percentage germination was only 5% after 14-week (3-month) storage and complete loss of viability was obtained at 21-week (4-month) storage period.

### C. Factors affecting spore germination

#### 1. Effect of temperature on spore germination

The effect of different temperatures on spore germination of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* was investigated and the results are given in Tables 6-8.

None of the temperatures stimulated spore germination of *F. fomentarius* on water agar. However, spores of *F. fomentarius* germinated on malt extract agar at all the temperatures. The influence of low temperature (15° C) upon germination rate of the spores was definite, since only 1% germination was obtained at 15° C. Above 20° C spore germination rate increased remarkably. Although there was not a definite difference





Table 4. Longevity of spores of *F. fomentarius*.<sup>a</sup>

Storage period (months)	Percentage germination
0	76%
1	76
3	55
4	18
5	15
6	0

a: Spores collected on April 28th, 1976.

Table 5. Longevity of spores of *F. cajanderi*.<sup>a</sup>

Storage period (weeks)	Percentage germination
0	41%
2	38
7	23
14	5
21	0

a: Spores collected on November 15th, 1976.



Table 6. Percentage germination of *F. fomentarius* spores on agar media at different temperatures.

Media	30 <sup>0</sup>	25 <sup>0</sup>	22 <sup>0</sup> (RT) <sup>a</sup>	20 <sup>0</sup>	15 <sup>0</sup> C
2% malt extract agar	66	72	75	69	1%
1% water agar	0	0	0	0	0

(RT)<sup>a</sup> = Room Temperature

Table 7. Percentage germination of *F. cajanderi* spores on agar media at different temperatures.

Media	35 <sup>0</sup>	30 <sup>0</sup>	25 <sup>0</sup>	22 <sup>0</sup>	20 <sup>0</sup>	15 <sup>0</sup>	10 <sup>0</sup> C
2% malt extract agar	32	34	29	33	28	25	23%
1% water agar	29	26	28	24	23	20	0

Table 8. Percentage germination of *F. igniarius* spores on agar media at different temperatures.

Media	35 <sup>0</sup>	30 <sup>0</sup>	25 <sup>0</sup>	22 <sup>0</sup>	20 <sup>0</sup>	15 <sup>0</sup> C
2% malt extract agar	11	16	14	14	8	0
1% water agar	13	12	12	9	1	0



in germination rate among the temperatures ( $20^{\circ}$ ,  $22^{\circ}$ ,  $25^{\circ}$  and  $30^{\circ}$  C), the spores incubated at  $22^{\circ}$  C showed a slightly higher germination rate than those at other temperatures.

*F. cajanderi* spores germinated in a broad range of temperatures on both media, except that no germination took place on water agar at  $10^{\circ}$  C. There was no remarkable difference in germination rate between 2% malt extract agar and 1% water agar. However, it should be noted that the germ tubes on 2% malt extract agar were generally longer and sturdier than those on 1% water agar. Also, the length of germ tubes was much longer (approximately 300 - 700  $\mu$ ) at higher temperatures ( $35^{\circ}$ ,  $30^{\circ}$ , and  $25^{\circ}$  C) than that of germ tubes (approximately 10 - 30  $\mu$ ) at lower temperatures ( $10^{\circ}$  and  $15^{\circ}$  C) on 2% malt extract agar.

Spores of *F. igniarius* germinated on both media at all the temperatures, except  $15^{\circ}$  C. Generally, the percentage germination was very low on both media, with the maximum of 16% on 2% malt extract agar at  $30^{\circ}$  C. Although the percentage germination at  $35^{\circ}$  C was slightly higher on 1% water agar than that on 2% malt extract agar, it was lower on 1% water agar than on 2% malt extract agar at other temperatures. There was no clear difference in the length of germ tubes between on 2% malt extract agar and on 1% water agar, nor among the temperatures.

## 2. Effect of substrate on spore germination

The effect of different substrates on spore germination of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* was examined and the percentage germination results are summarized in Table 9.

Spores of *F. fomentarius* germinated on all the woods tested: the highest germination rate was 85% on birch, the second highest was 79%





Table 9. Percentage germination of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* spores on different substrates.

	Birch		Aspen		Balsam Poplar		Spruce		2% malt	1%	Distilled
	wood	bark	wood	bark	wood	bark	wood	bark	extract	water	water <sub>a</sub>
									agar	agar	
<i>F. fomentarius</i>	85	0	79	0	63	0	67	18	51	0	0
<i>F. cajanderi</i>	49	14	47	0	32	0	42	40	35	36	6
<i>F. igniarius</i>	10	0	19	0	0	0	11	20	18	0 <sub>(+)</sub> <sub>a</sub>	0

0<sub>(+)</sub><sub>a</sub> : Less than 1% germination.



on aspen, the third was 67% on spruce, and the lowest rate was 63% on balsam poplar. All the germination rates were higher on woods than on 2% malt extract agar. On the other hand, the spores did not germinate at all on the intact bark of birch, aspen, and balsam poplar. The only bark on which germination took place was spruce with 18% germination.

*F. cajanderi* exhibited similar results, although the germination rate was generally lower than that of *F. fomentarius*. Spores of *F. cajanderi* also germinated on all the woods with the highest germination on birch and the lowest on balsam poplar. *F. cajanderi* showed spore germination on birch and spruce bark, but not on aspen and balsam poplar bark. The germination rate on spruce bark was almost as high as that on spruce wood. The spores germinated not only on 1% water agar, but also on distilled water, although the germination rate was very low.

The germination of *F. igniarius* on these substrates was a little different from those of *F. fomentarius* and *F. cajanderi*. The percentage germination was very low, with the maximum of 20% on spruce bark. The spores germinated on wood of birch, aspen, and spruce, but not on balsam poplar. No germination was observed on bark of birch, aspen, and balsam poplar.

#### D. Morphology and cytology of spore germination

##### 1. Morphology

###### 1) Light microscopy

Mode of spore germination of *F. fomentarius* and *F. cajanderi* was examined under the light microscope, and some cytoplasmic and nuclear



movements were observed with phase contrast and Nomarski interference microscope.

Spores of *F. fomentarius* did not swell prior to germ tube emergence. Germination usually took place at the apiculate end or the opposite end of the spores (Plate 1. A). Almost always a single germ tube was put out at one end of the spore and shortly after another was put out from the other end (Plate 1. A). Occasionally, germ tubes were put out simultaneously from both ends (Plate 1. A), but in general, one preceded the other. These observations show that *F. fomentarius* spores have bipolar germination. Very rarely, germination from the side of the spores occurred (Plate 1. B), and in this case, the emerging germ tubes were rather narrow and irregularly elongated. Bipolar germ tubes were not as wide as the original spores, but elongated rapidly, producing mostly unbranched hyphae. Some of the spores showed septum formation during germination and the spores often became 2-celled (Plate 1. C) or 3-celled after 24-hour incubation on 2% malt extract. Cytoplasm usually remained in the spores even after germ tube emergence, although some part of it moved into germ tube (Plate 1. C).

*F. cajanderi* showed a different mode of spore germination from that of *F. fomentarius*. Before putting forth germ tubes, spores of *F. cajanderi* swelled up to more than twice their original size (the ungerminated spores were approximately  $7.5 \times 2.8 \mu$ ; the swollen spores approximately  $11.1 \times 8.4 \mu$ ) (Plate 2. A). No definite polarity was observed in germ tube emergence and the swollen spores produced one or two germ tubes from the ends or sides in any direction (Plate 2. B). The germ tubes were nearly as wide as the swollen spores, and formed





a septum occasionally (Plate 2. C). Cytoplasm migrated toward the tip of germ tube so that the original spores became partially empty. On the dialysis membrane the majority of germinating spores formed an appressorium-like structure at the tip of germ tube (Plate 2. D). Nuclei tended to move with the cytoplasm into the germ tube (Plate 2. D).

## 2) Scanning electron microscopy

With the scanning electron microscope, spore surface features and mode of germination on wood tissues and agar medium were clearly seen.

The freeze-dried spores showed a comparatively smooth wall surface when they were ungerminated (Plate 3. A), but exhibited some shrinkage and a mucous-like substance on the wall surface of germinating spores (Plate 3. B). The shrinkage caused some ridge formation on the spore wall. The germ tubes on the agar sometimes penetrated into the agar, and a mucous-like substance appeared to be formed around them (Plate 3. C). These tendencies were most noticeable with *F. fomentarius* and *F. cajanderi*. Spores of *F. igniarius* did not show shrinkage.

The critical-point-dried spores looked quite different from the freeze-dried ones. Both ungerminated and germinated spores remained unshriveled except those of *F. cajanderi*, and they did not show any mucous-like substance, so that the spore surface looked smooth.

The surface features of the bipolar germ tube of *F. fomentarius* were clearly shown by means of the scanning electron microscope. At the base of germ tube, the spore wall became rough (Plate 4. A, B). Germ tubes elongated rapidly, particularly on wood tissues and showed occasional septum formation (Plate 4. B). The young hyphae formed in this fashion grew through the lumen of the wood cell and appeared to be attached to the cell wall (Plate 4. D). The hyphae were unbranched or



sparingly branched.

The light microscopic observation that spores of *F. cajanderi* swelled and then put out a germ tube in a random direction on both agar media and wood was confirmed by the scanning electron microscope (Plate 5. A, B). The germinated spores of *F. cajanderi* also exhibited septum formation in the germ tube, and partial depressions were common on the spore walls as well as on germ tube walls (Plate 5. C). The germination rate and mode of germination on spruce were not different from those on birch wood.

Spores of *F. igniarius* were round in shape with a nodulose apiculus (Plate 6. A). The spores showed a different mode of germination from those of the other two species. There was no spore swelling prior to germ tube emergence. Immediately after the emergence, the germ tube became much wider than its base, producing a constriction between the spore and the germ tube (Plate 6. B). Also, some of the germ tubes branched shortly after their emergence.

## 2. Cytology

### 1) Transmission electron microscopy

Ungerminated spores of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* and germinated spores of *F. fomentarius* and *F. cajanderi* were examined by means of transmission electron microscopy.

#### a. *Fomes fomentarius*

Basidiospores of *F. fomentarius* had a thick cell wall (approximately 340 nm) composed of two layers (Plate 7. A, B). The outer layer was electron-dense and amorphous. The thickness of the outer layer was thin (15 - 30 nm). The inner layer was a thick (310 - 325 nm), electron-light



and somewhat fibrous layer. The thickness of the inner layer was constant except for the area around the apiculus where the layer became thicker. Basidiospores of *F. fomentarius* did not have germ pores.

Before the spores produced germ tubes, the spore cell wall exhibited considerable changes. One of the first noticeable changes was that the outer layer became thicker and more electron-dense and, at the same time, the inner layer became more fibrous and electron-translucent. In addition, two more layers were newly formed inside the inner layer (Plate 8. A, B). Next to the inner layer, a very thin (approximately 9 nm), but electron-dense layer, and inside this, a relatively thick (approximately 50 - 70 nm), electron-light, fibrous layer were formed. In some spores septum formation was also observed at this stage. The septa were continuous with the newly formed innermost layer of the cell wall (Plate 9. A).

On initiation of germ tube emergence, the outer three layers of cell wall appeared to break down and allowed the passage of the germ tube (Plate 9. B). The innermost layer of the wall appeared extensible and formed the germ tube wall. As germination continued, some electron-dense material was deposited which formed a new thin layer around the germ tube wall (Plate 9. B). The germ tube eventually became a young hypha and the cell wall showed no further changes (Plate 10. A).

The cell membrane of ungerminated *F. fomentarius* spores was closely adpressed to the inner cell wall (Plate 10. B), but in germinated spores, it became more noticeable and highly invaginated on the side facing the cytoplasm. These invaginations occurred at irregular intervals (Plate 8. B).





Basidiospores of *F. fomentarius* were uninucleate. The nucleus with a nucleolus was surrounded by a nuclear envelope which exhibited well defined nuclear pores (Plate 11. A).

Mitochondria of ungerminated spores were poorly defined (Plate 10. B), although they were scattered in cytoplasm. They appeared as loosely arranged membranes or vesicles. However, during germination, mitochondria became more organized and distinguishable, exhibiting their membrane system with cristae more clearly. Some of the mitochondria were considerably elongated (Plate 8. A).

Typical rough endoplasmic reticulum (ER) was observed in ungerminated spores (Plate 11. B). The ER was fairly elongated, sometimes forming lamellae. As germination proceeded, the ER could not be observed.

Many lipid bodies were found in cytoplasm of ungerminated spores (Plate 7. A). They were spherical in shape and relatively electron-light. In germinating spores, the number of lipid bodies seemed to decrease and the lipid bodies appeared to become more electron-translucent (Plate 7. A, Plate 9. A).

No vacuoles were observed in ungerminated spores of *F. fomentarius*. However, vacuolization took place during the germination process (Plate 8. A, Plate 9. A and B). Small vacuoles were a usual feature of early germination, but later, they enlarged by fusing. In addition, numerous electron-dense, amorphous inclusions were observed in the vacuoles. They were found mostly within or in close proximity to the tonoplast. (Plate 8. A, Plate 9. A).

Numerous ribosomes were observed in both germinated and ungerminated spores. Some microbody-like organelles were found in germinated spores. A membrane complex was also observed in germinated spores (Plate 9. B).



b. *Fomes cajanderi*

The cell wall of *F. cajanderi* spores was quite different from that of *F. fomentarius*. It consisted of only one layer which was very thin (approximately 70 nm), and electron-light (Plate 12. A and B). The spore wall appeared quite extensible, and when the spore germinated, the wall elongated to form the germ tube wall (Plate 12. C). Therefore, no breakage in the original spore wall was observed. Some of the germinated spores showed septum formation in which the septum wall was continuous with the spore wall.

The cell membrane of ungerminated spores was generally inconspicuous (Plate 12. B), while that of germinated spores was relatively noticeable. Slight invagination of the cell membrane was also observed in germinated spores, but the degree of invagination was less than that of *F. fomentarius* germinated spores.

In ungerminated spores, nuclei were not clearly observed, while they were distinguishable in germinated spores (Plate 12. C, and Plate 13. A). Homogeneous nucleoplasm as well as condensed nucleoli were recognized, although the nuclear envelopes were not clear.

As was the case with *F. fomentarius* spores, mitochondria of *F. cajanderi* ungerminated spores were not well defined. They were scattered in the relatively condensed cytoplasm (Plate 13. B). During germination, the mitochondria became more transparent than the surrounding cytoplasm, and the number appeared to increase (Plate 12. C).

The presence of ER was also recognized in the ungerminated spores of *F. cajanderi* (Plate 13. C). This ER seemed to disappear during germination.



Some lipid bodies were found in *F. cajanderi* ungerminated spores (Plate 13. D), but the number of them appeared to be fewer than observed in *F. fomentarius*. In germinated spores, even fewer lipid bodies were observed.

In ungerminated spores vacuoles were not found, but in some of them, small, membrane-bounded vesicles were occasionally observed (Plate 13. D). However, whether these were related to vacuoles or not was not clear. When the spores were germinating, typical vacuoles were formed with electron-dense inclusions (Plate 14. A). The size of the vacuoles and the inclusions increased until some of these inclusions occupied a considerable volume of the cell (Plate 14. B).

The cytoplasm was relatively condensed and numerous ribosomes were observed. A myelin figure was also found (Plate 12. C). Occasionally, spore degeneration was observed. The degenerating spores were characterized by scanty cytoplasm which showed some reticulate arrangement (Plate 14. C).

### c. *Fomes igniarius*

The cell wall of *F. igniarius* ungerminated spores was similar to that of *F. fomentarius*. It had a thick wall (approximately 580 nm) composed of two layers: the electron-dense outer layer was approximately 80-100 nm in thickness and the more or less electron-light inner layer was approximately 480-500 nm (Plate 15. A). The outer layer was amorphous and the inner layer was somewhat fibrous.

The cell membrane, nucleus, and ER were poorly defined in ungerminated spores because of condensed cytoplasm.

Lipid bodies were relatively larger and more abundant in ungermin-





ated spores of *F. igniarius* than those of *F. fomentarius*.

Spore degeneration was also observed, and was characterized by aggregation of the cytoplasm into small portions. The degeneration eventually resulted in loss of cytoplasm, lipid bodies, and mitochondria (Plate 15. B).

The electron microscopic (and light microscopic) observations are summarized in Table 10.

## 2) Light microscopy with histochemical techniques

Spores of *F. fomentarius* embedded in three plastic media were examined under the microscope after the application of various staining methods, and the cytological changes during germination were observed.

In all the embedding media used the form of fungal spore cells was well preserved and there was no apparent interference from the embedding media in the quality of the image. Although the affinity of each embedding medium to various stainings was compared, all the media showed similar reactions, and the difference in the degree of stain penetrations among the three was not distinctive.

The results obtained from the observation are summarized in the Tables 11-15.

Characteristic colors of Toluidine Blue (T.B.) on *F. fomentarius* spores were obtained with 1% Toluidine Blue in 1% Borax, but not with the 0.05% T.B. in Benzoate buffer. The cell wall stained dark bluish purple and was readily distinguished from the slightly bluish purple of the cytoplasm. The nuclei, which showed a homogeneous, blue content, were clearly differentiated from the cytoplasmic portion of cells (Plate 16. A). The nucleolus appeared dark bluish purple. Vacuoles remained



Table 10. Summary of morphological and cytological changes of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* spores during germination.

	<i>F. fomentarius</i>	<i>F. cajanderi</i>	<i>F. igniarius</i>
Spore swelling	-	+	-
Germ pore	-	-	-
Germ tube emergence	bipolar	irregular	irregular
Cell walls	thick, 2-layered ↓ 4-layered	thin, 1-layered ↓ 1-layered	thick, 2-layered
Cell membrane	not clear ↓ clearer, invaginated	not clear ↓ clearer	not clear
Nuclei	uninucleate ↓ dividing	uninucleate ↓ dividing	not clear
Mitochondria	not clear ↓ clearer	not clear ↓ clearer	not clear
ER	present ↓ not clear	present ↓ not clear	not clear



Table 10. (continued)

	<i>F. fomentarius</i>	<i>F. cajanderi</i>	<i>F. igniarius</i>
Storage products	many lipid bodies ↓ less	a few lipid bodies ↓ less	abundant lipid bodies
Vacuoles	absent ↓ present, with inclusion	absent ↓ present, with inclusion	absent





Table 11. Effects of Toluidine Blue (pH 8.0) on thick sections of *F. fomentarius* spores embedded in different plastic media.

	Spurr (Germinated spores)	Epon (Germinated spores)	Araldite (Ungerminated spores) (Germinated spores)
plastic	unstained	unstained	unstained
cytoplasm	slightly bluish purple	slightly bluish purple	slightly bluish purple
nucleus	blue	blue	blue
nucleolus	dark bluish purple	dark bluish purple	dark bluish purple
lipid body	unstained	unstained	purple
vacuole	unstained	unstained	unstained
vacuole inclusion	dark red	dark red	stained but not clear
cell wall	dark bluish purple	dark bluish purple	dark bluish purple



Table 12. Effects of Periodic acid-Schiff on thick sections of *F. fomentarius* spores embedded in different plastic media.

	Spurr (Germinated spores)	Epon (Germinated spores)	Araldite (Ungerminated spores)	(Germinated spores)
plastic	unstained	unstained	unstained	unstained
cytoplasm	unstained	unstained	unstained	unstained
nucleus	slightly pink	slightly pink	slightly pink	slightly pink
nucleolus	unstained	unstained	unstained	unstained
lipid body	reddish pink	reddish pink	slightly pink	reddish pink
vacuole	unstained	unstained	not present	unstained
vacuole inclusion	reddish	reddish	not present	reddish
cell wall	pinkish red	pinkish red	pinkish red	pinkish red



Table 13. Effects of IKI on thick sections of *F. fomentarius* spores embedded in different plastic media.

	Spurr (Germinated spores)	Epon (Germinated spores)	Araldite (Ungerminated spores)	(Germinated spores)
plastic	unstained	unstained	unstained	unstained
cytoplasm	unstained	unstained	unstained	unstained
nucleus	unstained	unstained	unstained	unstained
nucleolus	unstained	unstained	unstained	unstained
lipid body	unstained	unstained	dark-colored	dark-colored
vacole	unstained	unstained	not present	unstained
vacuole inclusion	dark brown	dark brown	not present	dark brown
cell wall	unstained	unstained	unstained	unstained



Table 14. Effects of Sudan Black B on thick sections of *F. fomentarius* spores embedded in different plastic media.

	Spurr (Germinated spores)	Epon (Germinated spores)	Araldite (Ungerminated spores)	(Germinated spores)
plastic	slightly orange	slightly orange	slightly orange	slightly orange
cytoplasm	slightly orange	slightly orange	slightly orange	slightly orange
nucleus	slightly orange	slightly orange	slightly orange	slightly orange
nucleolus	slightly orange	slightly orange	slightly orange	slightly orange
lipid body	slightly orange	slightly orange	dark red	dark red
vacuole	slightly orange	slightly orange	not present	slightly orange
vacuole inclusion	slightly orange	slightly orange	not present	dark-colored
cell wall	slightly orange	slightly orange	slightly orange	slightly orange





Table 15. Unstained thick sections of *F. fomentarius* embedded in different plastic media.

	Spurr (Germinated spores)	Epon (Germinated spores)	Araldite (Ungerminated spores) (Germinated spores)
plastic	unstained	unstained	unstained
cytoplasm	unstained	unstained	unstained
nucleus	unstained	unstained	unstained
nucleolus	unstained	unstained	unstained
lipid body	unstained	unstained	dark-colored
vacuole	unstained	unstained	not present
vacuole inclusion	unstained	unstained	not present
cell wall	unstained	unstained	slightly dark-colored
			unstained



unstained, while the vacuole inclusions appeared as dark-red bodies (Plate 16. B). Lipid bodies were not observed in Spurr and Epon sections, but found to be stained purple in Araldite sections (Table 11).

With Periodic acid-Schiff (PAS) stain, cytoplasm, vacuole, and nucleolus did not appear stained, although nuclei showed a slightly pinkish color (Plate 16. C). The cell wall of both germinated and ungerminated spores stained brilliant pinkish-red (Plate 16. C). PAS reacted with the lipid bodies more intensively in germinated spores than in ungerminated spores. The PAS positive reaction in these bodies was as prominent in Epon- and Spurr-embedded materials as in Araldite-embedded ones. Vacuole inclusions in germinating spores showed reddish color with this stain (Table 12).

The cell organelles were not stained with IKI, except that some vacuole inclusions stained dark brown. This indicates that the inclusions had some reactivity with IKI (Table 13).

Only the lipid bodies of the spores embedded in Araldite stained dark-red with Sudan Black B (Plate 16. D). On the other hand the lipid bodies in Spurr-and Epon-embedded materials were not stained (Table 14).

In unstained thick sections embedded in Araldite, lipid bodies showed a dark color, and vacuole inclusions were slightly dark colored (Table 15).

With these staining techniques, some of the cytological changes during spore germination of *F. fomentarius* were shown clearly. PAS reacted with cell wall, giving a brilliant pinkish-red color, and T.B. also reacted with the cell wall, showing dark bluish purple. However, none of the staining procedures could differentiate the inner layer from the outer one of the spore cell wall. The cytoplasm of the



ungerminated spores was relatively dense, as shown in T.B. stained sections. During germination, the cytoplasm moved towards the germ-tube and was replaced by vacuoles. A nucleus, with a nucleolus in it, was suspended in the center of cytoplasm of each ungerminated spore. During germination, the nucleus divided into two, one daughter nucleus remained in the spore, and the other migrated toward the germ-tube. Vacuoles were not found in ungerminated spores, but once the spores started germinating, vacuoles appeared in the spores as well as in the germ tubes. In the early stage of spore germination the vacuoles seemed small in size, but later became larger. Concomitant with vacuole formation, some inclusions, which stained with T.B., PAS, and IKI, were being formed in vacuoles. The vacuole inclusions studied in this investigation clearly exhibited a different staining nature from that of lipid bodies. Numerous lipid bodies were dispersed in ungerminated spores as seen in Sudan Black B-stained sections. It seemed that the number of lipid bodies decreased during germination. Some lipid bodies appeared to move into the germ tubes.







Plate 1. Spores of *F. fomentarius* germinating on dialysis membrane on malt agar.

- A) Bipolar germination: a single germ tube emerging from an apiculate or an opposite end of the spore (1); a second germ tube emerging later from the opposite end (2); two germ tubes emerging almost simultaneously from both ends (3).

Phase contrast X 1000.

- B) An exceptional mode of germination with a germ tube emerging from side of the spore (arrow).

Phase contrast X 1200.

- C) Septa (SE) and nucleus (N) in germinating spores.

Interference contrast X 2100.

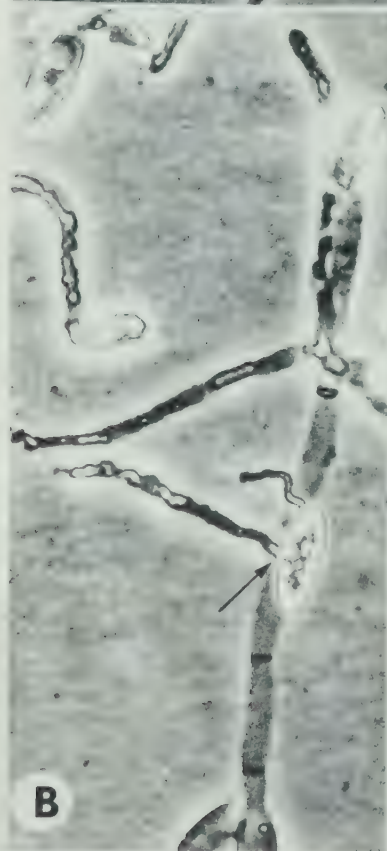
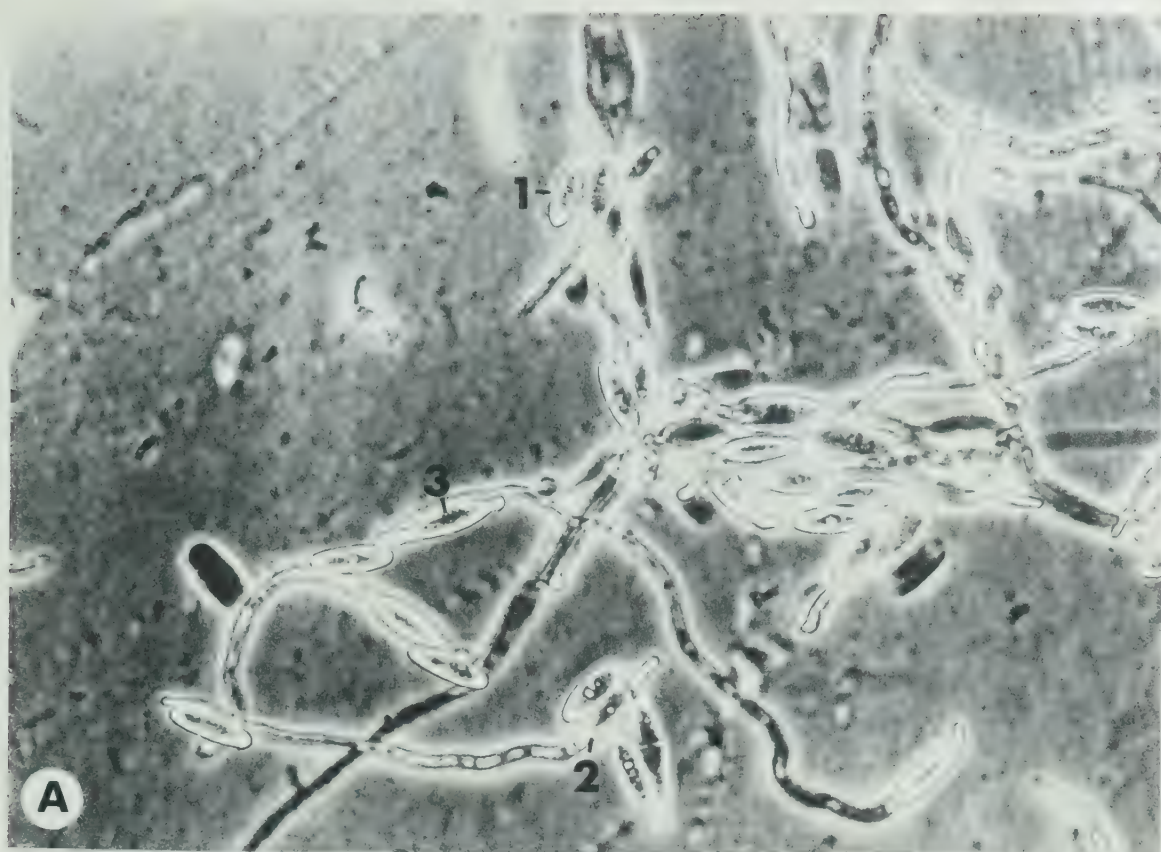






Plate 2. Spores of *F. cajanderi* germinating on dialysis membrane on malt agar.

- A) Unswollen, ungerminated spores (1), swollen, ungerminated spore (2), and germinating spores (3).  
Phase contrast X 1600.
- B) Germinated spores. One or two germ tubes (G) emerge at random sites of spore (S).  
Phase contrast X 1600.
- C) A germinated spore with a septum (SE).  
Interference contrast X 2800.
- D) Germinated spores in which cytoplasm (C) and nucleus (N) of the spore (S) have migrated into the germ tube (G).  
The tip of the germ tube has become swollen to form an appressorium-like structure (A).  
Interference contrast X 3000.



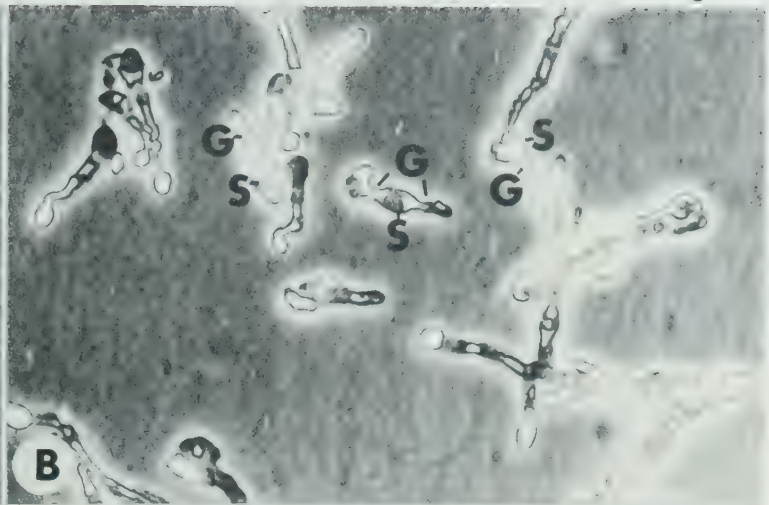
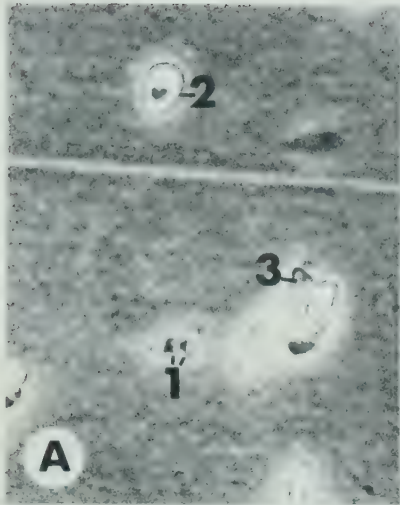








Plate 3. Spores of *F. fomentarius* prepared with freeze-drying method.

A) Ungerminated spores showing comparatively smooth wall surface.

SEM X 2500.

B) Germinated spores with some shrinkage effects.

Ridge (R) formation is evident on the spore surface.

SEM X 2500.

C) Germinated spore with a germ tube penetrating the agar medium. The germ tube appears covered with mucous-like substance.

SEM X 3300.

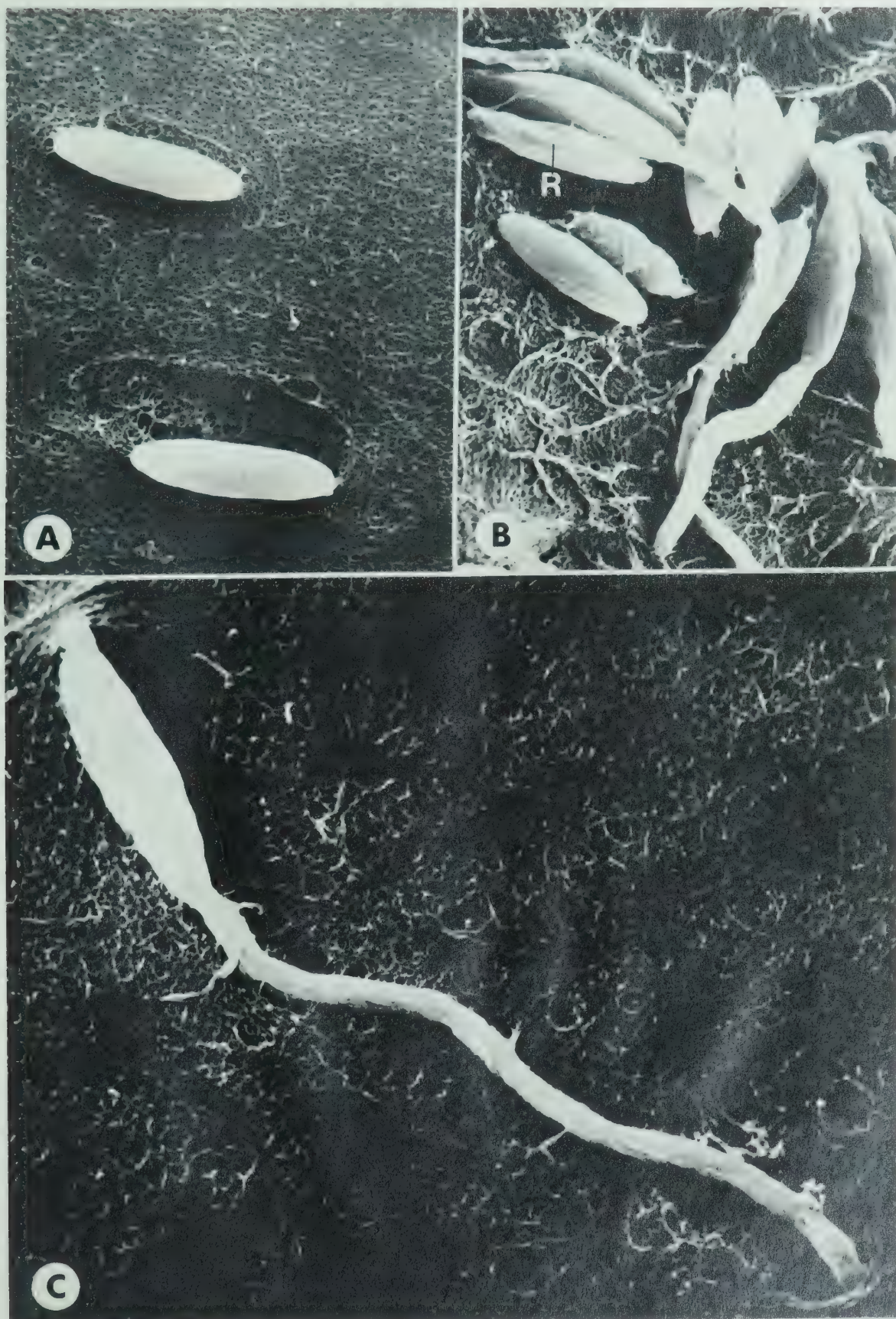








Plate 4. Spores of *F. fomentarius* prepared with critical-point drying method.

A) Initial stage of spore germination. The wall surface of the spore is smooth and there is no shrinkage. The cell wall appears to be rough and broken at the germination site (arrow).

SEM X 6000.

B) Septal formation (arrow) in a germ tube. The cell wall surface is rough at the base of the germ tube.

SEM X 15000.

C) Elongation of germ tubes on spruce wood. Typical bipolar germination is shown.

SEM X 1500.

D) Young hyphae growing through the lumen of a wood cell. Note hyphal branches (arrows) which are closely appressed to the wood surface.

SEM X 1000.



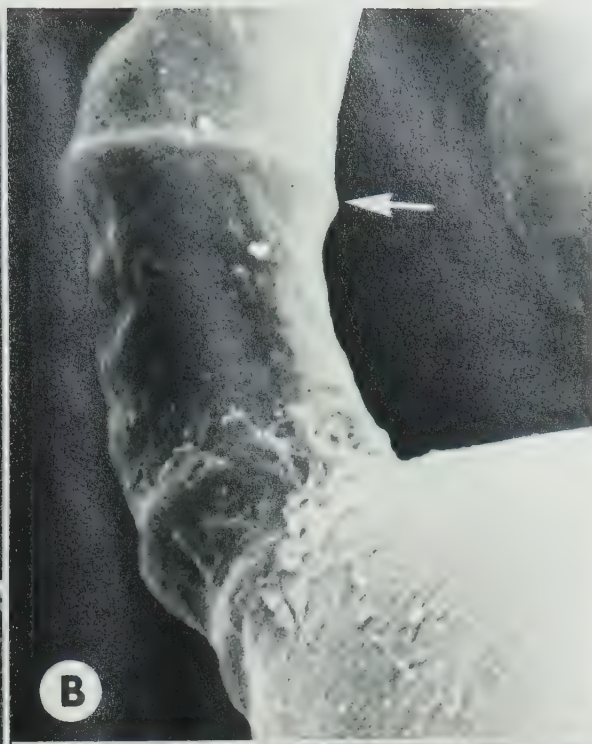






Plate 5. Spores of *F. cajanderi* prepared with critical-point drying method.

A) Germinated spores on malt agar with germ tubes (G) emerging at random sites of the spore surface.  
SEM X 3400.

B) Unswollen (1) and germinating spores (2) on spruce wood.  
SEM X 4000.

C) Germinated spore with depressions (D) on the spore (S) wall and on the germ tube (G) wall.  
SEM X 10000.











Plate 6. Spores of *F. igniarius* on malt agar.

A) Ungerminated spores prepared with freeze-drying method.

Note the nodulose apiculi (AP).

SEM X 8600.

B) Germinated and ungerminated spores prepared with critical-point drying method. Note the presence of a constriction (CN) in the germ tubes at the emerging area. The precipitation covering the materials was formed during dehydration procedure because of the use of the impure alcohol.

SEM X 4000.

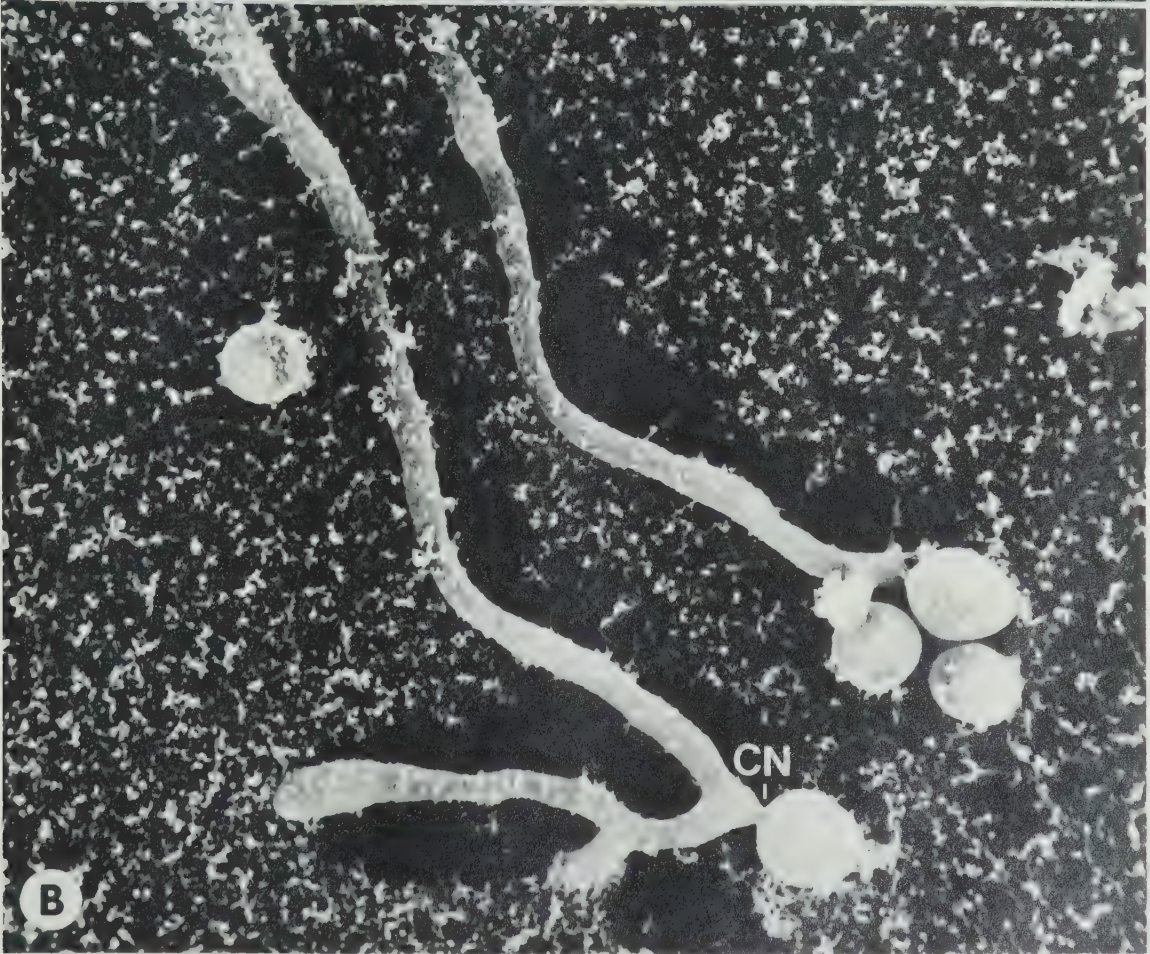
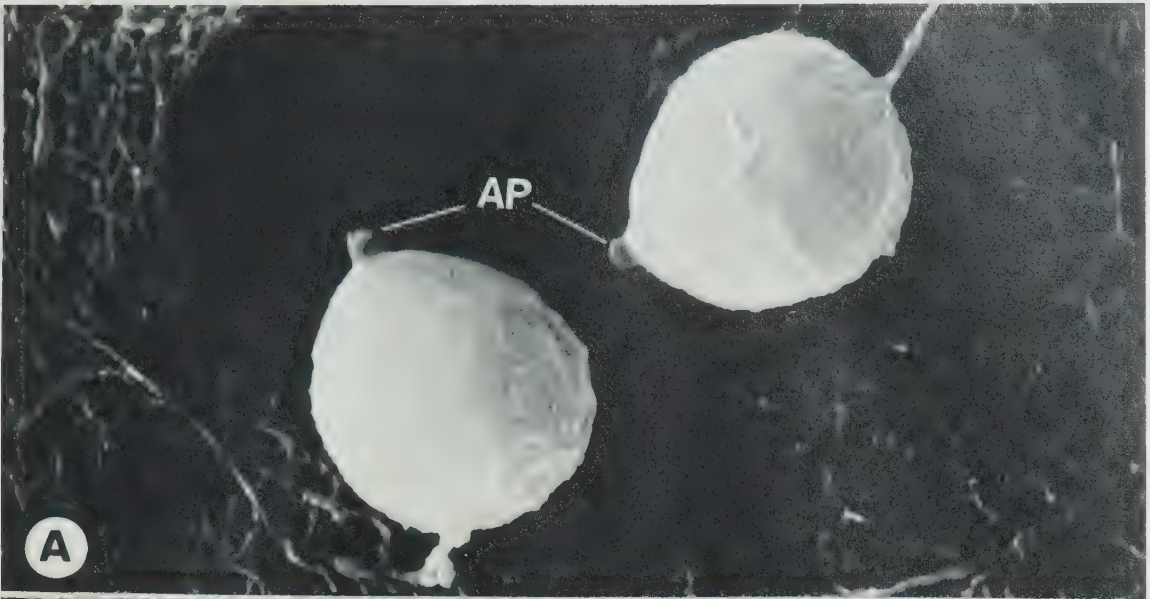






Plate 7. Ungerminated spores of *F. fomentarius*.

- A) Cross section of an ungerminated spore. Note the thick cell wall (CW), endoplasmic reticulum (ER) and many lipid bodies (L).

TEM X 20000.

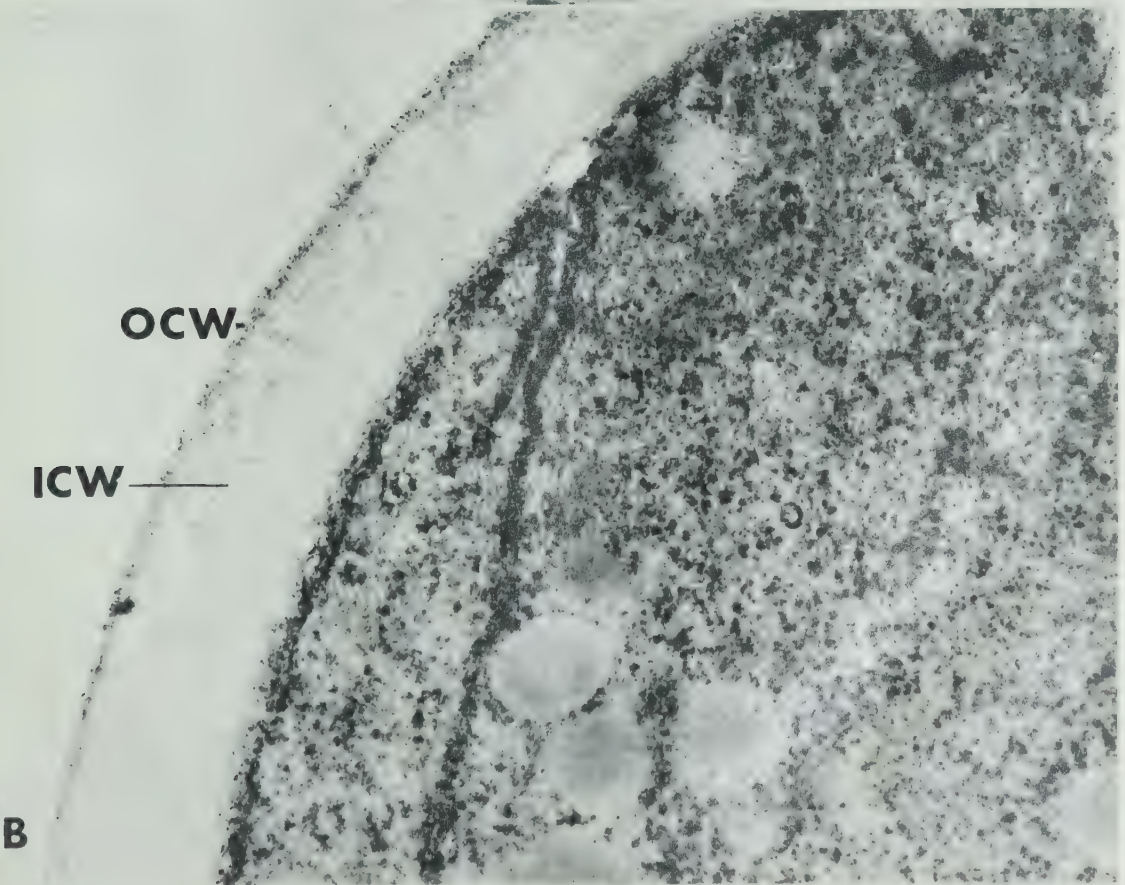
- B) Magnified view of Plate 7-A). The cell wall consists of two layers: an electron-dense amorphous outer layer (OCW) and an electron-light inner layer (ICW).

TEM X 70000.





**A**



**B**







Plate 8. A germinated spore of *F. fomentarius*.

- A) Cross section of a germinated spore. In comparison with the ungerminated spore (Plate 7, A), the cell wall shows greater differentiation and there are fewer lipid bodies (L). Formation of large vacuoles (V) with electron-dense inclusions (VI) and microbody-like organelle (MB) occurs. Mitochondria (M) can be clearly seen.

TEM X 43000.

- B) Magnified view of Plate 8-A). The cell wall consists of four layers: an outermost electron-dense amorphous layer (CW<sub>1</sub>); an electron-light, fibrous, thick layer (CW<sub>2</sub>); an electron-dense, very thin, newly formed layer (CW<sub>3</sub>) and an innermost electron-light, newly formed layer (CW<sub>4</sub>). Invaginated plasma membrane (PM) is also clear.

TEM X 97000.

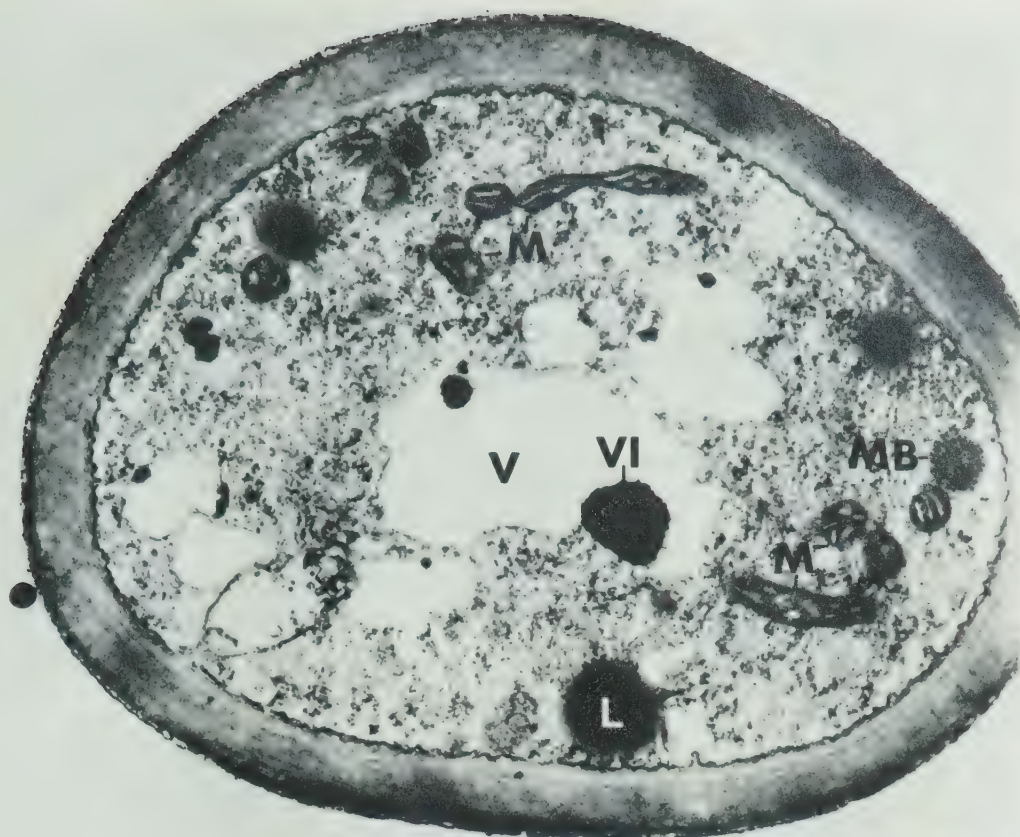
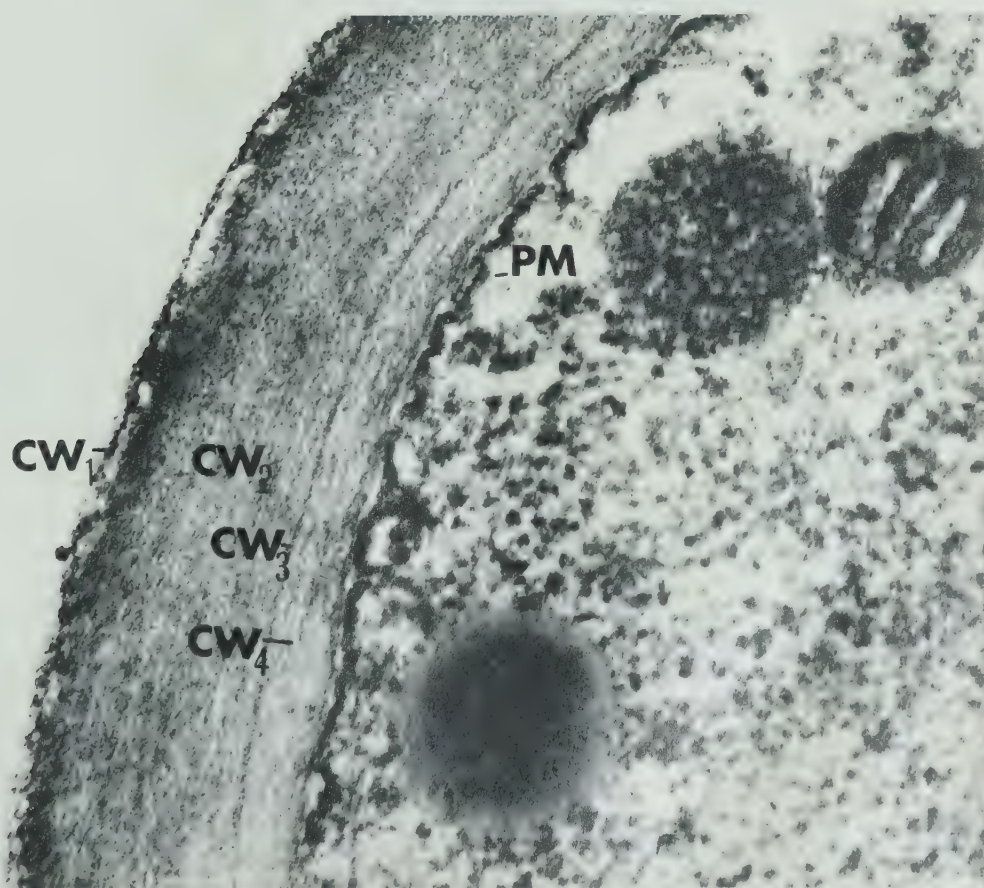
**A****B**







Plate 9. Germinated spores of *F. fomentarius*.

A) Longitudinal section of a germinated spore. Note the dissolution of the outer layers of the cell wall on initiation of germ tube emergence (arrow). The electron-light, innermost layer is continuous with the newly formed septa (SE).

TEM X 12000.

B) Longitudinal section of a germinated spore and its germ tube (G). The outer layers of the cell wall are broken at the base of germ tube (arrows). The innermost layer of the cell wall of the spore is continuous with the wall of germ tube. New wall material (WM) is deposited around germ tube. Membrane complex (MF) is seen.

TEM X 11000.

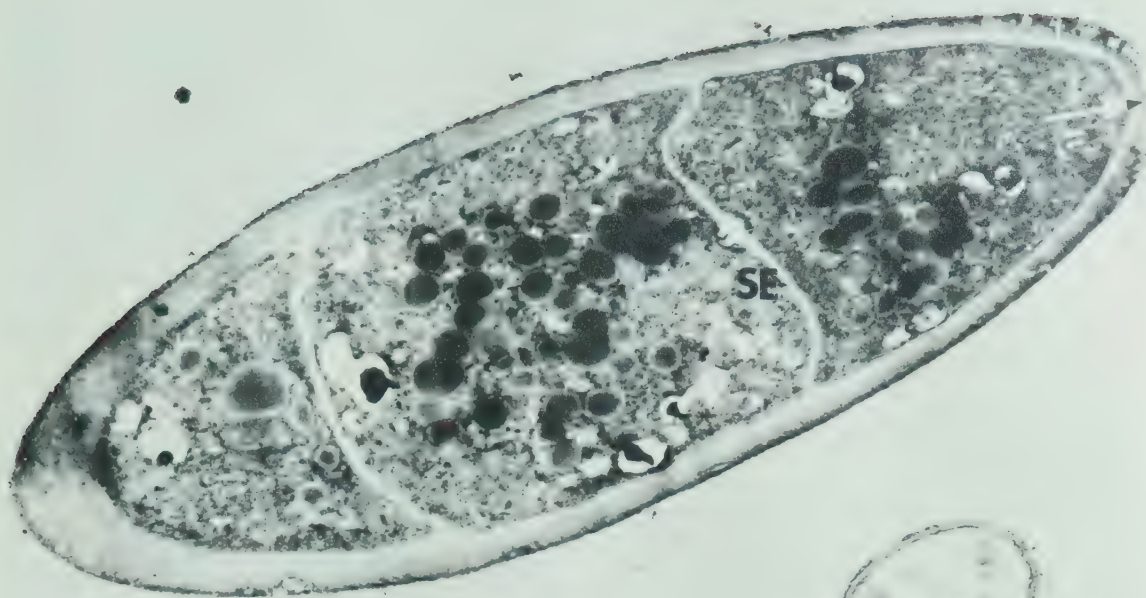
**A****B**





Plate 10. An elongated germ tube and an ungerminated spore of  
*F. fomentarius*.

A) An elongated germ tube with a thin cell wall.

TEM X 22000.

B) Part of a longitudinal section of an ungerminated  
spore. Note the relatively inconspicuous plasma  
membrane (PM) and mitochondria (M).

TEM X 20000.



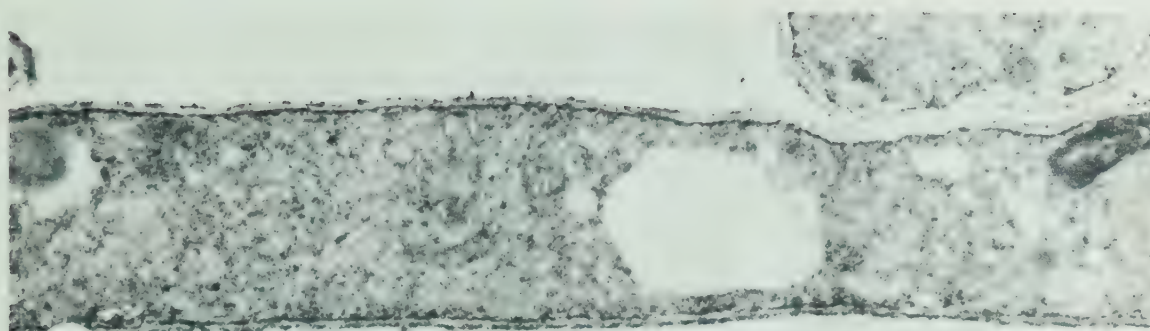
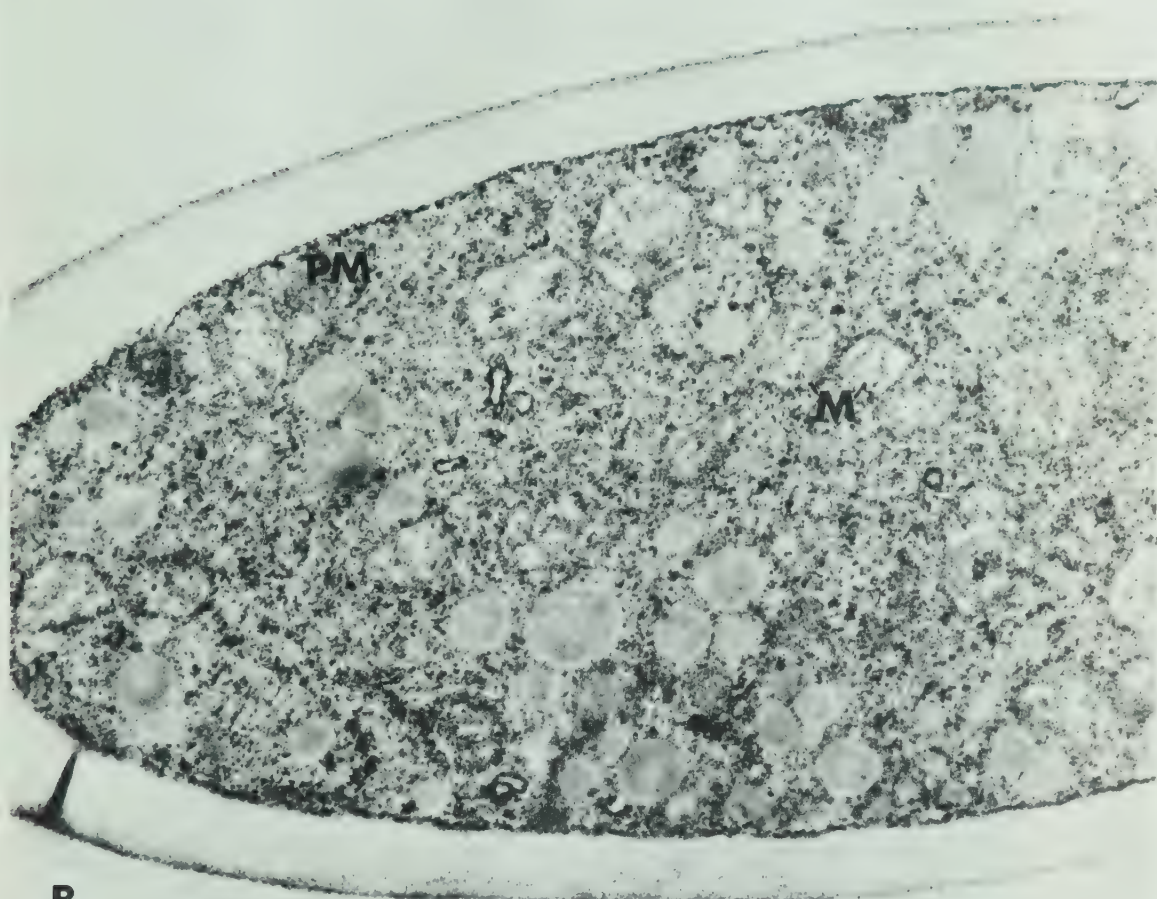
**A****B**







Plate 11. Ungerminated spores of *F. fomentarius*.

- A) Cross section of an ungerminated spore, showing a nucleus (N) with a nucleolus (NO) and nuclear pores (NP).

TEM X 17000.

- B) Cross section of an ungerminated spore. Note the conspicuous lamellae of the endoplasmic reticulum (ER).

TEM X 15000.

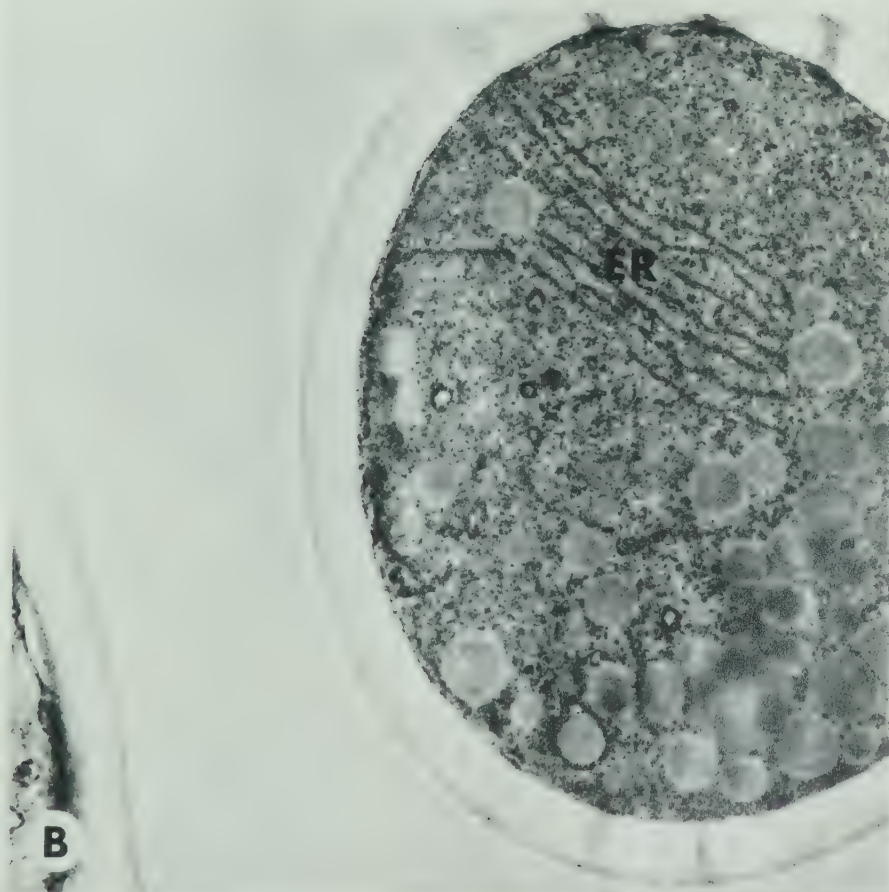
**A****B**







Plate 12. Ungerminated and germinated spores of *F. cajanderi*.

- A) Longitudinal section of ungerminated spore.  
TEM X 19000.
- B) Magnified view of a portion of the ungerminated spore. Note presence of very thin, electron-light cell wall (CW). TEM X 70000.
- C) Longitudinal section of germinated spore. The cell wall of the germ tube is continuous with the cell wall (CW) of the spore. A nucleus (N), predominant mitochondria (M), and a myelin figure (MF) are seen. TEM X 15000.

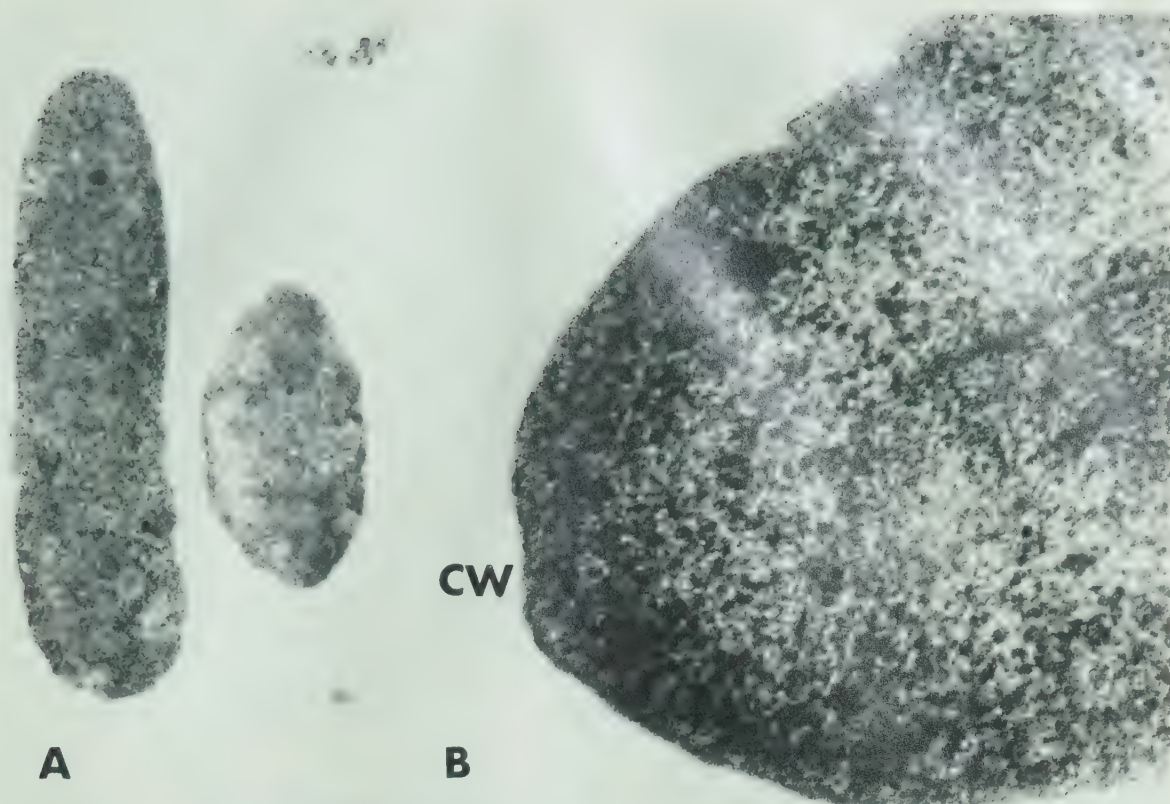






Plate 13. Germinated and ungerminated spores of *F. cajanderi*.

- A) Cross section of a germinating spore, showing a large nucleus (N) with a nucleolus (NO). The plasma membrane (PM) is seen.

TEM X 22000.

- B) Part of a longitudinal section of an ungerminated spore with mitochondria (M) in relatively condensed cytoplasm.

TEM X 73000.

- C) Cross section of an ungerminated spore. Note the presence of extensive endoplasmic reticulum (ER).

TEM X 44000.

- D) Longitudinal section of an ungerminated spore.

Lipid bodies (L) and a membrane-bound vesicle (VE) are clearly seen.

TEM X 87000.



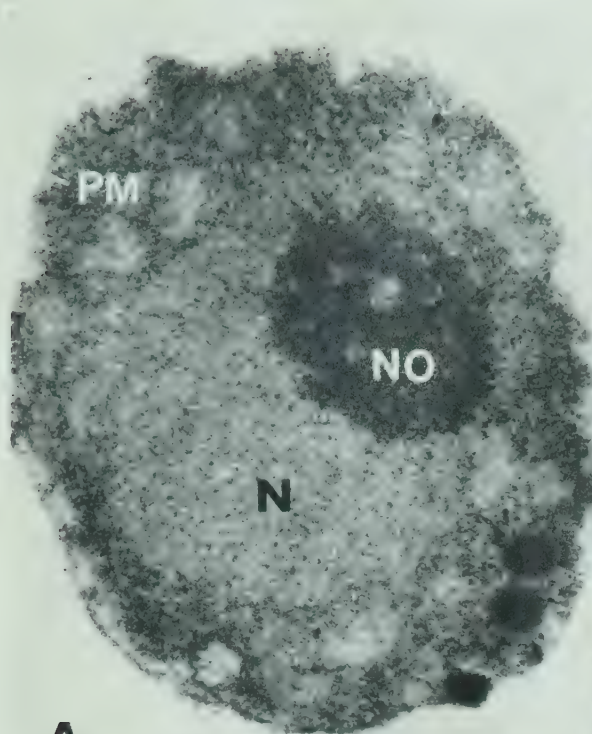
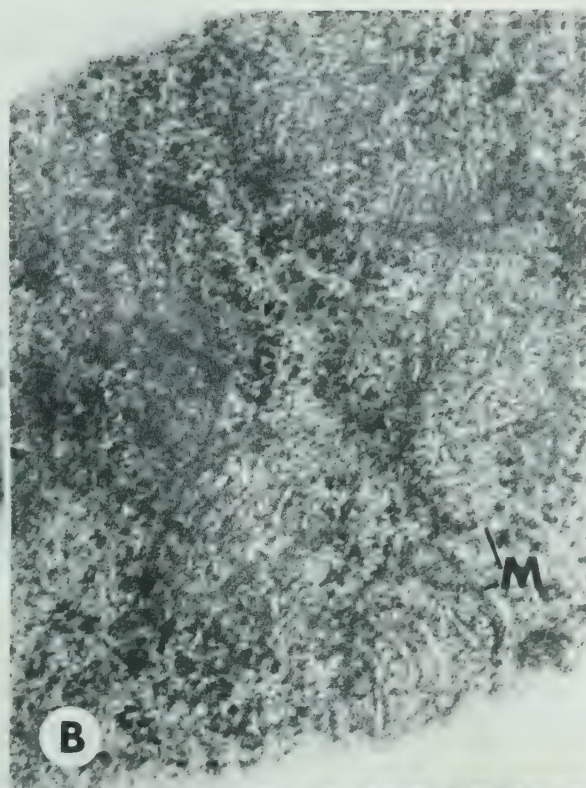
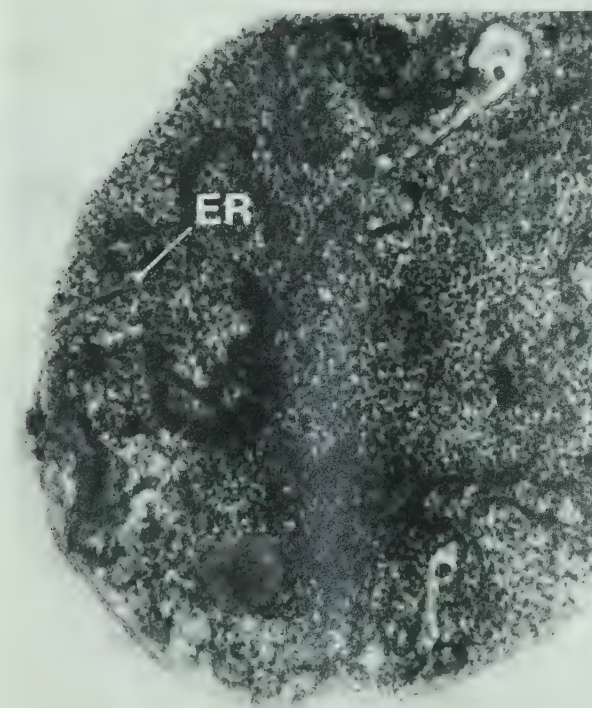
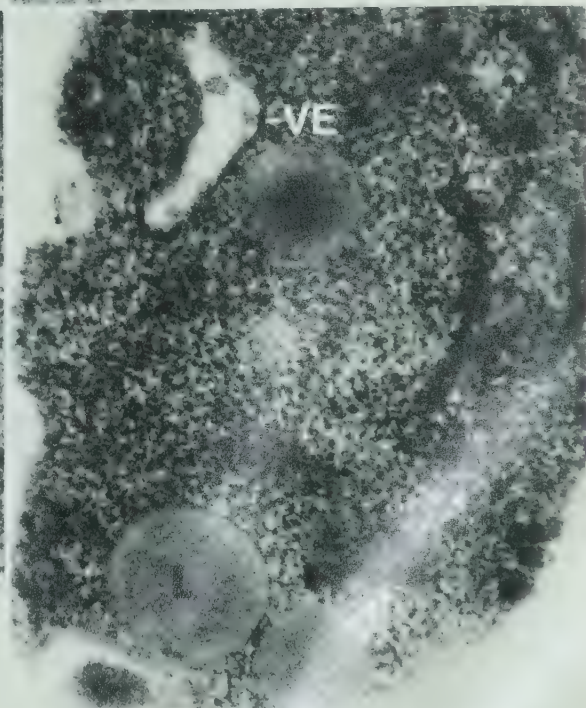
**A****B****C****D**







Plate 14. Germinated and degenerated spores of *F. cajanderi*.

- A) Cross section of a germinated spore, exhibiting large vacuoles (V) with electron-dense inclusions (VI).

TEM X 32500.

- B) Longitudinal section of a germinated spore with a large vacuole inclusion (VI) in the vacuole.

TEM X 14000.

- C) Part of a longitudinal section of degenerating spore.

Note the scanty cytoplasm showing reticulate arrangement (arrow).

TEM X 77000.

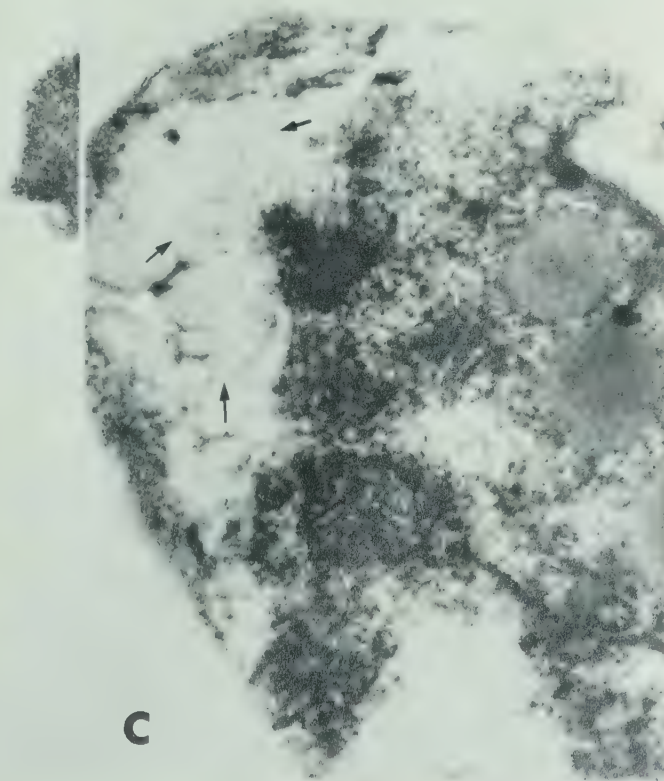
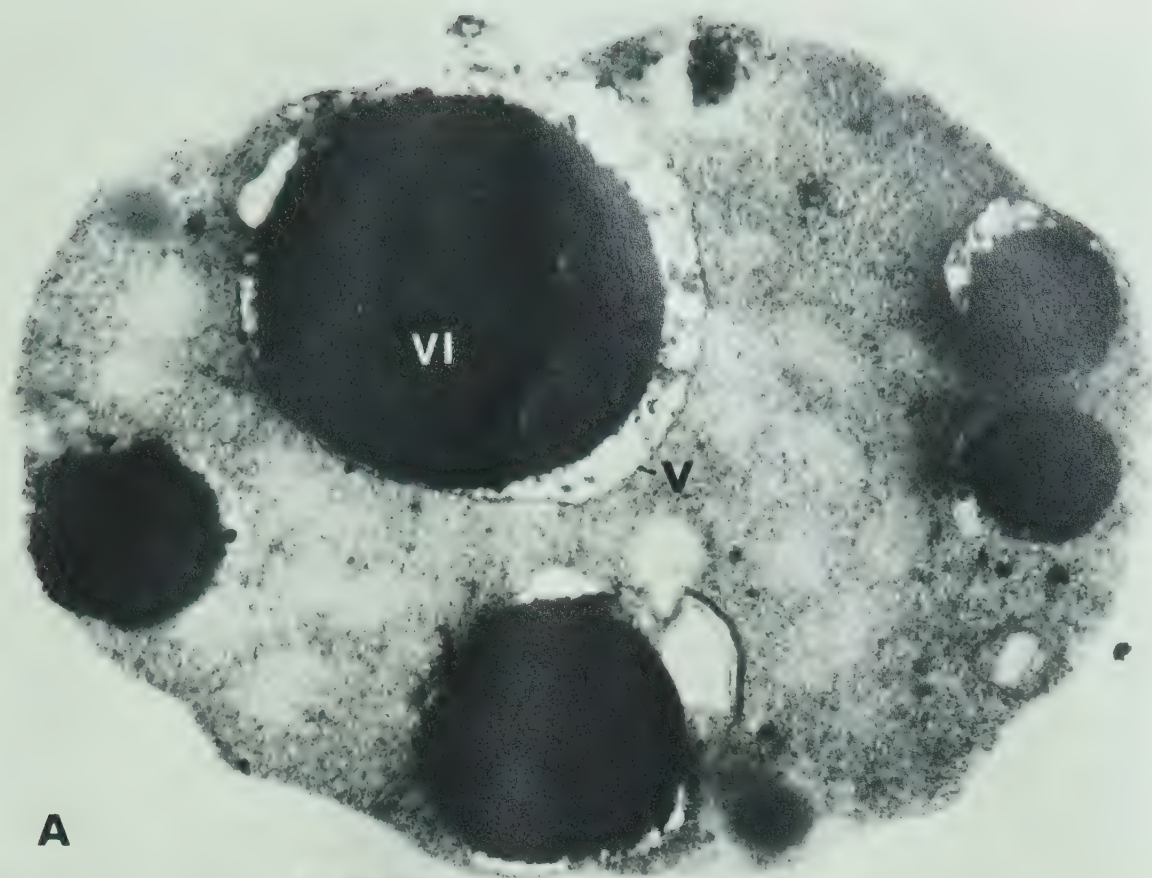








Plate 15. Ungerminated spores of *F. igniarius*.

- A) A cross section, showing a thick cell wall with an electron-dense outer layer (OCW) and an electron-light inner layer (ICW). Note predominant lipid bodies (L) scattered in condensed cytoplasm.

TEM X 40000.

- B) A cross section of a degenerating spore. Scanty cytoplasm (C) and degenerating mitochondria (M) are evident.

TEM X 42000.

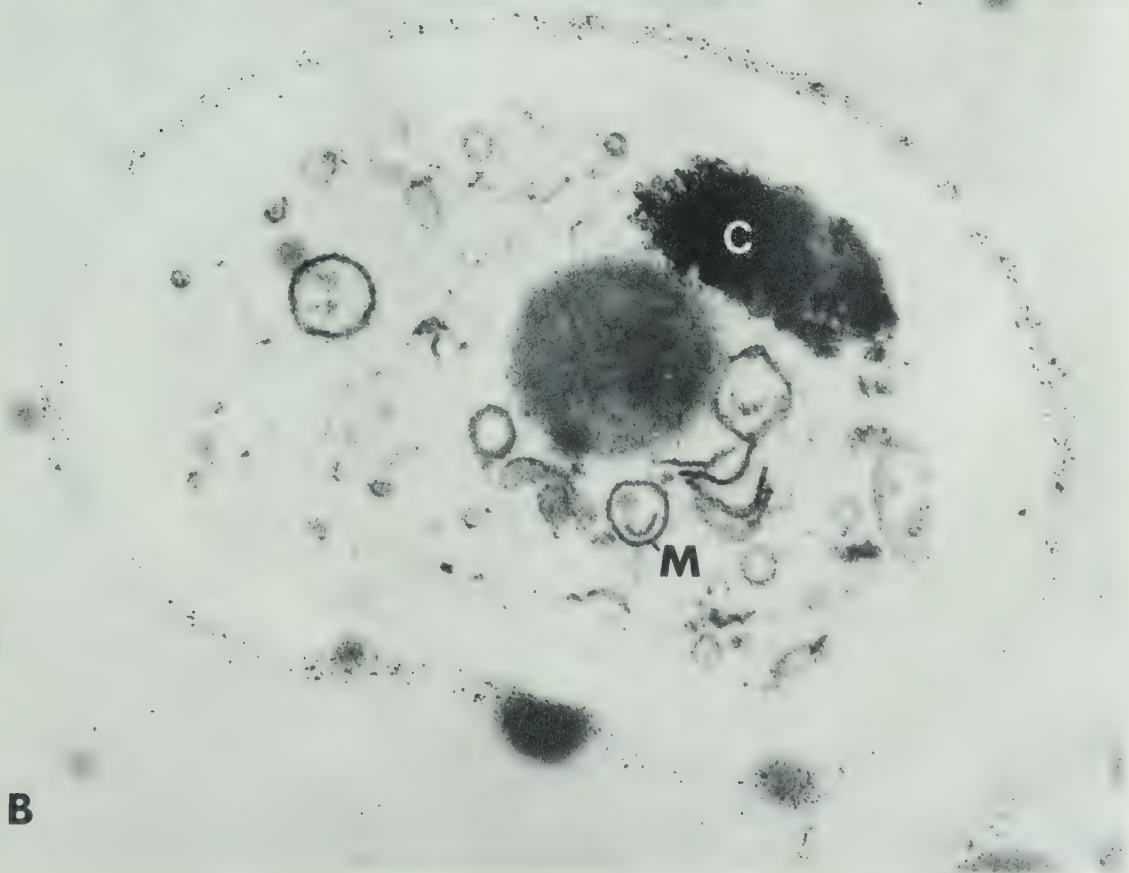
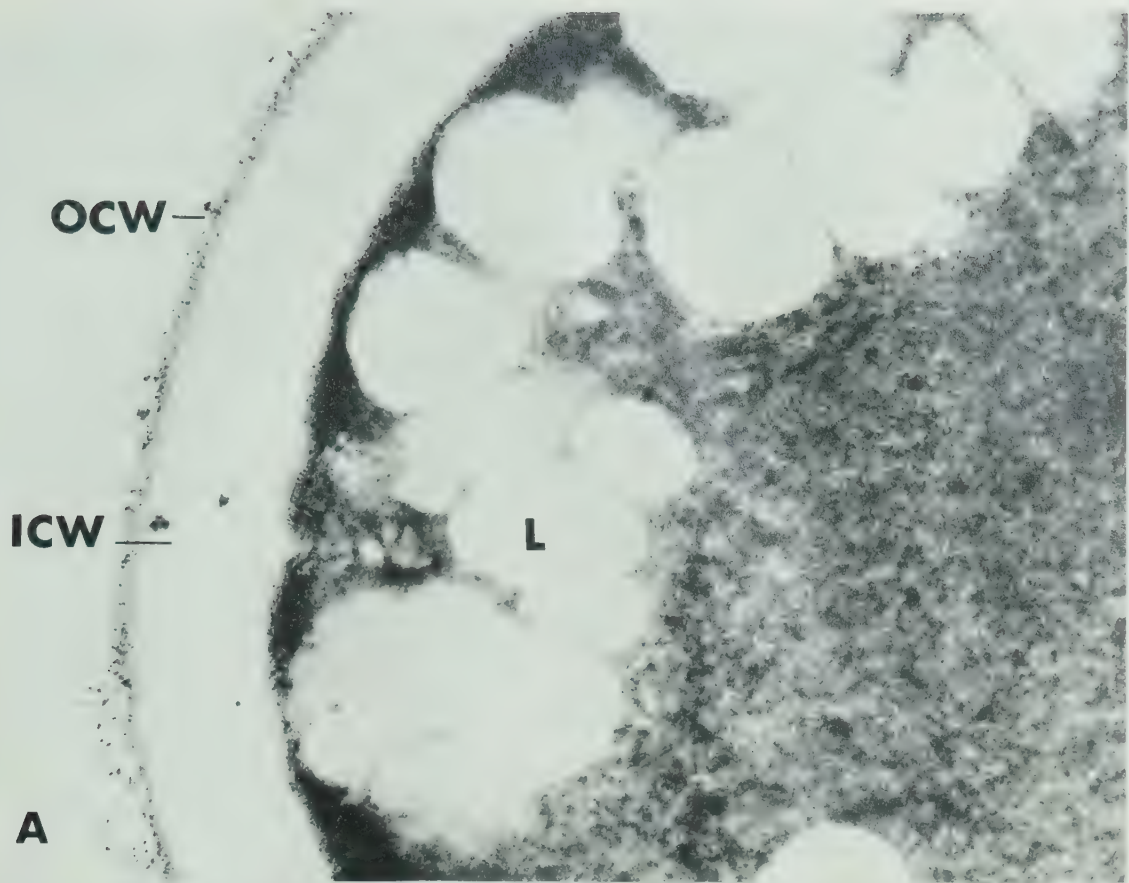






Plate 16. Germinated spores of *F. fomentarius*.

- A) Longitudinal section, stained with 1% Toluidine Blue.  
Note a nucleus (N) and many vacuoles (V).  
Phase contrast X 2000.
- B) Longitudinal section, stained with 1% Toluidine Blue.  
Dark stained vacuole inclusions (VI) in vacuoles (V)  
are evident.  
Phase contrast X 2000.
- C) Longitudinal section, stained with Periodic-Acid-Schiff.  
The nuclei (N), lipid bodies (L) and cell wall (CW)  
are visible.  
Phase contrast X 2000.
- D) Longitudinal section, stained with Sudan Black B.  
Dark-colored lipid bodies (L) are clear.  
Phase contrast X 2000.







## DISCUSSION

In the present investigation, several aspects of spore germination of *F. fomentarius*, *F. cajanderi* and *F. igniarius* were studied. The main objectives were to determine the effects of selected factors influencing spore germination and to examine morphological and cytological changes of the spores during germination. The following discussion will deal with: spore sampling, spore germinability, spore longevity, factors affecting spore germination, and morphology and cytology of spore germination.

### A. Spore sampling

The uniformity of spore collections as an experimental material is important for the study of spore germination. In this study, since *F. fomentarius* and *F. igniarius* did not form their sporocarps on the cultural medium used, the spores of these species were collected under natural conditions. These spores presented several problems. Mixed contaminations were unavoidable, there were always seasonal variations in spore productivity, and this field material was inherently variable in germinability. Since these variations were considerable, appropriate precautions were required in collecting, handling and germinating spores of these fungi. In order to overcome these difficulties, experiments were repeated until relatively constant data were obtained. Since *F. cajanderi* formed sporocarps on the medium, both spores collected in the field and in culture were used. The spores of *F. cajanderi* collected under natural condition usually had the contamination problem while those from culture medium were free from other microorganisms. The results in germination were relatively constant when the latter



spores were used. Thus, most of the germination tests were conducted using the spores collected from culture.

#### B. Germinability of spores

According to Manner (1966), the germination process includes three stages: (a) Internal changes, i.e., morphological and physiological changes; (b) Germination, i.e., the act of protrusion of the germ tube from the spore wall; (c) Germ-tube growth, i.e., the elongation of the germ tube. In spite of these definitions, it is difficult to devise methods whereby the progress of each stage may be measured accurately and rapidly as a routine procedure. Therefore, most workers have preferred to employ an arbitrary criterion based on the appearance of an easily visible germ tube. There are several suggestions for the assessment of germination. The American Phytopathological Society (1943) stated: "the spore is arbitrary defined as germinated if the length of the tube exceeds half the minor diameter of the spore". Other workers (Manner 1966; French 1961; Manner and Hossain 1963) have preferred to consider a spore as germinated when the germ tube is as long as it is broad. For the assessment of spore germination, it is most important to use consistent methods, especially when comparisons are made. Therefore, in the present study, the latter definition was preferred, since it represents the earliest stage of the *Fomes* species, at which one can be certain that a spore is germinated, using rapid examination under the light microscope. Employing this arbitrary criterion, spore germination of the three species of *Fomes* was examined.

It is generally known that rapidity of spore germination and percentage germination vary in different fungal species. In this study



these aspects of the *Fomes* species were investigated. From the results, it is clear that the spore of *F. fomentarius* is a relatively fast germinator, requiring about 8 hours to initiate germination and germinating in most cases within 24 hours. Similar observation was made with *F. cajanderi* spores. On the other hand, *F. igniarius* showed relatively slow spore germination. This observation confirms the results with *F. igniarius* spores obtained by Good and Spanis (1958) who also reported that these spores took several days to initiate spore germination. It appears that the overall rapidity of spore germination is a species characteristic, for example, spores of *Melampsora lini* begin germination in less than one hour (Hart 1926).

When percentage germination was determined by periodic counts, each species showed a different percentage germination under the same experimental conditions. *F. fomentarius* showed relatively high spore germination (67%) after 24-hour incubation, as Meyers (1936) mentioned, though she did not indicate an exact percentage. The percentage germination of *F. igniarius* was low (14%) after 7-day incubation. On the other hand, Good and Spanis (1958) as well as Manion and French (1969) reported that the spores of *F. igniarius* exhibited a high percentage germination (over 90%). The difference in the germination between these studies and the present investigation may be due to the different media used, and/or to the different spore sources.

### C. Longevity of spores

Although in this study the spores collected were usually used as soon as deposited, some of them had to be stored, since there was no constant spore supply under natural conditions. It was, therefore,





necessary to determine the period of spore viability.

Records of the viability of many fungal spores have been reported and indicate a great difference in survival potential, but always a gradual decrease of viability with time. The decrease is dependent both on the inherent characteristics of the organism (Roberg 1948) and upon environmental conditions Doran (1922). Harrison (1942) examined the longevity of the spores of many wood-destroying Hymenomycetes and reported that longevity varied to a great extent among the different genera, or species, from the shortest of *Pleurotus ostreatus* (20 days) to the longest of *F. pinicola* (173 days). This variation in longevity is reported even among different spore collections of the same species. Good and Spanis (1958), in their extensive investigation of spore germination of *F. igniarius*, stated that the longevity of the spores varied from a few hours or days to 80 days, depending on the spore collections and also on the conditions in which spores were collected. In the present study, it was found that *F. fomentarius* spores lost viability after 6 months, although Meyer (1936) reported that spores of the same species showed 25% of germinating power one year after collection. Under natural conditions, there are several factors influencing survival of spores. Generally, those conditions which increase the metabolism of the organisms such as high temperature and humidity do not favor spore longevity: conversely low temperature and humidity increase the life span of spores because of the conservation of their stored food reserves. The investigations on *Polyporus pargamenus* (Rhoads 1918) and on *P. tomentosus* (Whitney 1966) are typical examples which provide evidence for this statement. However, in this study, only one spore collection of each species kept on a dry slide at



room temperature was tested to determine the longevity. It is necessary to examine more spore samples before a definite conclusion on spore longevity of the *Fomes* species is drawn. In addition, under natural conditions, a wide fluctuation of environmental conditions commonly takes place. Thus, the effects of fluctuating conditions of the environment on longevity should be examined.

It should be noted that since the spores of *F. fomentarius*, *F. cajanderi*, and *F. igniarius* were able to germinate under favorable conditions as soon as the spores were liberated from the sporocarps, it can be said that spores of these *Fomes* species have exogenous dormancy.

#### D. Factors affecting spore germination

During the spore germination period, a fungus is most dependent upon and susceptible to the influences of the physical, biochemical, and biological factors of the environment. Spores require suitable conditions for germination, involving such factors as temperature, moisture, pH, radiation, aeration, nutrients or substrates, and sometimes, the presence of other microorganisms. These factors play important roles in spore germination and may determine the preference of the *Fomes* species for certain kinds of wood. Among these factors, temperature and nutrients or substrates are especially critical.

The purpose of the study reported in this section was to determine the effect of temperature on spore germination and the effect of substrates on spore germination.

The effect of temperature upon germination has received more attention than any other environmental factor, due to its indirect influence upon the infection of economic plants by fungi. Not only is the percentage of germination affected, but also the time for germination



and the rate of elongation of the germ tubes. However, very little is known about the effect of temperature upon spore germination of wood-decay fungi, especially in species of the genus *Fomes*.

Among the limited number of studies, White (1919) reported that no acceleration or retardation in spore germination of *F. applanatus* was observed by altering temperature conditions between 10° and 27° C. Similarly, in the present study, it was found that spores of *F. cajanderi* germinated at a wide range of temperatures, 10° - 35° C, without a definite optimum temperature. But there was a difference in germ tube length at the different temperatures, that is the higher the temperature, the more the germ tubes elongated.

It is thought that different species show different optimum temperatures. According to Mounce (1929), spores of *F. pinicola* germinated at temperatures ranging from 8° - 35° C. A temperature of 8° C greatly retarded germination, temperatures from 22 - 30° C gave both good germination and good mycelial development, while 35° C caused a noticeable retardation in mycelial growth. This type of response to different temperatures was also observed with *F. fomentarius* in the present study. The highest percentage germination of the spores occurred at 22° C, and spore germination was somewhat retarded at other temperatures. The spores appeared to be very sensitive to the temperatures below 15° C, as percentage germination dropped to great extent. Although the type of response to different temperatures was not so definite as that of *F. fomentarius*, spores of *F. igniarius* showed some reaction to different temperatures. The spore germination of *F. igniarius* was almost equally rapid at temperatures of 22°, 25°, 30° and 35° C, with the maximum percentage germination at 30° C, but the low temperature of 15° C retarded the





spore germination. This observation is similar to the results obtained by Good and Spanis (1958), except that they reported that spore germination of *F. igniarius* was retarded at 10° C, instead of 15° C.

Thus, there are differences among the three *Fomes* species studied, in the response of the spores to temperature. Each species appears to have a different optimum temperature for spore germination as well as a different temperature tolerance. In general, the three species showed higher percentage germination at temperatures above 20° C which suggests that the most efficient and rapid spore germination of these species may take place in the warmer season in this area. Also, the ability to germinate in wide range of temperatures indicates that spores of these species are capable of germination at almost any temperature encountered during the growing season. This may provide more chances for survival under natural conditions for these species.

Since the constitution of the media was thought to alter the effect of temperature on spore germination, 2% malt extract agar and 1% water agar were used at the same time to observe such an effect. Spores of *F. fomentarius* did not germinate at all on 1% water agar at any temperature, therefore, such an effect may be negligible for this species. On the other hand, spore germination of *F. cajanderi* was retarded on 1% water agar only at the lowest temperature tested. In addition, *F. igniarius* showed slight retardation in spore germination on 1% water agar in the lower temperature range (15° - 22° C). This evidence suggests that the lower temperatures tend to retard spore germination of *F. cajanderi* as well as *F. igniarius* in the absence of enough nutrients, while the higher temperatures appear to stimulate the spore germination of these species, even if sufficient nutrients are not present.



Spores of many fungi contain sufficient reserves to allow respiration but in some species these reserves are insufficient to allow germination to proceed to the formation of germ tubes. In such cases, the spores require addition of exogenous nutrients or substrates to complete the process. Therefore, nutrients or substrates play a very important role in influencing spore germination. However, very few studies on nutrient requirements for spore germination of species in the genus *Fomes* have been made and variable results have been reported. The variation in results may be attributable to species preference for exogenous nutrients and different methods employed in the various studies.

The common methods of examining spore germination of wood-decay fungi may be divided into three groups: (1) liquid or agar media with known nutrients, (2) hot- or cold-water extracts of wood, and (3) intact wood as a nutrient source.

As an example of the first type of nutrient source, White (1919) stated that malt extract was the most satisfactory for spore germination of *F. applanatus*. All three *Fomes* species used in the present study showed spore germination on 2% malt extract agar. Spores of *F. fomentarius* required the presence of external nutrients for germination, since they did not germinate in distilled water or on water agar. Although Merrill (1970) reported spore germination of *F. fomentarius* on distilled water or water agar, results of the present study show that this species did require external nutrients. Merrill's results could be explained by the presence of some growth substances in the agar (Robbins 1939), and/or contaminating microorganisms in the spore source of *F. fomentarius*, which might provide some nutrient for *F. fomentarius*. In the preliminary experiment of this study, spores of *F. fomentarius*



did germinate on the water agar prepared with Difco agar, although the percentage germination was less than that on malt extract agar. In subsequent experiments, therefore, purified agar, 'Oxide' Agar (BDH), was used to prepare agar media. Spore germination of *F. igniarius* was also better on 2% malt extract agar than on water agar. *F. igniarius* appears to require some external nutrients, although the degree of the requirement may not be so high as for *F. fomentarius*, since the spores could germinate on water agar. This observation is similar to the results obtained by Good and Spanis (1958) who reported that spores of *F. igniarius* germinated best on 8% malt extract medium, and only a trace of germination occurred on water agar. On the other hand, *F. cajanderi* spores differed in their nutrient requirement from that of the other two species. The spores germinated on water agar and distilled water as well as on 2% malt extract agar, which indicates that they did not require external nutrients for germination. Similar observations have been made by others with spores of *F. roseus* (Morton 1964) and *F. cajanderi* (Merrill 1970). Thus, it can be said that each species has different nutrient requirements. Although these investigations indicate increased germination of spores of *Fomes* species by the nutrients in malt extract, it is doubtful if the fungus spores would encounter such luxurious substrates in nature. Experiments using a substrate more like those occurring in the natural environment would give more useful information.

Since wood extracts are considered to play an important role in determining whether fungal spores germinate on a particular substrate, the effects of wood extracts on spore germination have been investigated. Mounce (1929) examined spore germination of *F. pinicola* by using hot-water decoction of pine wood and obtained no germination. This may have





been due either to the breakdown of substances essential for germination, or to the formation of toxic products in the decoction (Morton and French 1966). It can be concluded that hot-water extracts or decoctions of wood may not be suitable as a nutrient source. On the other hand, cold-water extracts of wood appear to be effective in stimulating spore germination. Good germination of *F. igniarius* spores was obtained by using cold-water extracts of wood (Good and Spanis 1958; Wall and Kuntz 1964). A similar observation was made by DeGroot (1965) who reported 80% germination of *F. pini* spores on media containing cold-water extracts of pine wood. In spite of the fact that wood extracts would be expected to be an ideal natural nutrient source for spore germination, there are some problems in using them. Since there is no standardized method to prepare wood extracts, various methods have been used by different investigators. Consequently, the concentration of the different extracts as well as nutrient composition may differ, and the results obtained may not be suitable for comparison. The use of a standard method for the preparation of cold-water extracts is recommended.

In order to overcome these disadvantages of wood extracts, wood has been used directly to observe the effects of substrate tissues as a nutrient source for spore germination. Price (1913) was the first to describe the spore germination of *Polyporus squamosus* on wood or wood strips. Meyer (1936) reported that spores of *F. fomentarius* germinated rather well on water-soaked blocks of wood, although the precise methods were not clearly described in these early studies. Morton and French (1966) used microtome sections of nonextracted Douglas-fir sapwood and reported a higher percentage germination for *F. roseus* spores on them. But in this case it appears unsatisfactory to use microtome sections for



spore germination, since they are usually too thick to be examined under a light microscope. However, the techniques for observing spore germination directly on wood surfaces have been developed and more promising methods can be now used. As a most sophisticated method, scanning electron microscopy will be mentioned, although this method is not handy for routine work, since more elaborate procedures are involved. Another method is the Sellotape impression method. By impressing the adhesive side of a tape to wood, spores on the wood are collected and examined under a light microscope. Although this method is commonly used in plant pathology, especially, when phylloplane microorganisms are to be examined, it is not adequate for woody tissues which, unlike leaves, usually have rough surfaces. The third method is to use discs of dialysis membrane directly on the wood surface (Manion and French 1969). By removing the discs from the wood surface, it is possible to observe the membrane under a light microscope and to examine spore germination more precisely than with the Sellotape impression method. Therefore, in this study the membrane method was selected to examine the effect of different kinds of wood and bark on spore germination. Since a dialysis membrane, which might create artifacts, was placed between spores and wood tissues, the reliability of this method was not certain. But when the result obtained by this method was compared with that obtained by scanning electron microscopy, the germination percentages were very close and correlated. It can be concluded that this membrane method is reliable.

In the present study, it was assumed that the preferences of wood-decay fungi for certain kinds of woods might be attributable to the ability of the spores to germinate on the woods or their bark, or attributable to a higher degree of germinability of the spores on those



substrates than on others. To test this assumption, the spore germination of three *Fomes* species was examined on the bark and wood of birch, aspen, balsam poplar, and spruce. On the bark of spruce spore germination of the three *Fomes* species was observed, although no spores of the species germinated on aspen or balsam poplar bark. This suggests that the spores of the *Fomes* species may have preference for spruce. Yet, from the results of preliminary study, both aspen and balsam poplar bark were shown to contain more sugars and amino acids than birch and spruce bark (Appendix 11). Apparently aspen and balsam poplar bark have some inhibitory effect on spore germination of these fungi. Indeed, Hubbes (1964, 1966, 1969) demonstrated the presence of fungus inhibitory compounds in aspen bark. Spore germination of *F. fomentarius* was observed on all the kinds of wood. Since the percentage germination on wood of birch and aspen was higher than on balsam poplar and spruce, it can be said that *F. fomentarius* spores exhibited a slight preference for birch and aspen. In contrast, *F. cajanderi* showed spore germination to a similar extent on all the woods. Similarly, spore germination of *F. igniarius* was not particularly stimulated by any of the woods, so that no definite preference was evident. It appears that substrate-preference of these species is not due to the inability of their spores to germinate on non-substrate wood, nor is it due to higher percentage germination on a particular type of wood. It seems that substrate-preference of these *Fomes* species is controlled by factors other than those influencing spore germination. It is considered that substrate-preference may be determined by factors which control the subsequent colonization and growth of these fungi in the wood. Similar results were obtained by Paine (1968) even with *Polyporus betulinus* which is





a typical host-specific fungus, occurring only on *Betula* species. Paine reported that spores of *P. betulinus* germinated on non-host species of *Abies*, *Larix*, *Picea*, *Pinus*, and *Tsuga*. Later, Paine and Merrill (1971) suggested that host specificity of this species was controlled by factors other than those acting to prevent spore germination and subsequent colonization. Paine (1968) stated that the normal microflora inhabiting dead coniferous branch stubs did not retard spore germination, but stimulated it. Later, Paine and Merrill (1971) suggested that host-specificity was due partially to factors which control growth of these competitors. Therefore, competitive ability with other microorganisms, and/or adaptability to the specific environmental condition of a particular substrate may play important roles in determining substrate-preference by wood-decay fungi. Further investigation of these aspects is required in order to explain substrate-preference of wood-decay fungi.

Little is known about germination-site phenomena of wood-decay fungi. Rhoads (1918) noted that there must be some condition conducive to infection or penetration, such as broken branch, or other form of wound or a drying out and consequent death of the cambium as a result of fire. It is thought that fungi cannot gain entrance unaided through the bark which envelops the entire living trunk, since fungal germ tubes are incapable of forcing their way through layers of cork. However, this commonly accepted view has not been demonstrated, and especially, the possibility of bark as a germination-site has never been examined. In this study, the possibilities of both wood and bark as germination-sites were investigated. The results clearly demonstrated that spores of the three species almost always gave much better germination on wood than on



bark. This indicates that wood is indeed a more desirable germination site than bark. However, since spore germination of all the species took place on spruce bark, and spores of *F. cajanderi* germinated on birch bark, although to a lesser extent than on wood, the possibility of bark as a germination site cannot be ignored. But it is doubtful if subsequent hyphal penetration and establishment in the bark takes place, since further examination after spore germination did not show this.

It is now clear that wood is a more suitable site for spore germination than bark. However, the effect of the freshness of the wood on spore germination is not known. In a natural forest stand, there are woods in various states - from freshly exposed, to partially decayed by microorganisms, to totally decayed. Which of these states is most suitable for spore germination of wood-decay fungi is not understood. In the present study, only freshly cut, sound wood and bark were used, and a stimulative effect of freshly cut wood on spore germination was observed. Especially, spore germination of *F. fomentarius* was stimulated by all the wood, even better than by 2% malt extract agar. This observation suggests that the freshly cut wood may contain a more appropriate combination of nutrients than 2% malt extract agar and provide the fungal spore ideal conditions for germination. A similar observation was made by Meredith (1959) who reported that fresh wood or uncolonized wood may be more suitable than colonized wood. In contrast, it has been reported that partially decayed woods do not retard spore germination of wood-decay fungi, but stimulate it (Good and Spanis 1958; Wall and Kuntz 1964; DeGroot 1965; Paine 1968). Since there is contradictory information, the effect of the condition of wood on spore germination must be more thoroughly examined.



## E. Morphology and cytology of spore germination

In the previous section, it was pointed out that spores of *F. fomentarius*, *F. cajanderi* and *F. igniarius* germinated when the spores encountered a suitable environment, but that each species showed different responses to various environmental factors. Particularly, the differences in nutrient requirement were evident among the three species. It was found in the preliminary study that these species exhibited different morphological features and changes during spore germination. Therefore, the external morphology and internal structure and organization of the ungerminated and germinated spores were compared.

The external features of spores were more clearly shown with scanning electron microscope (SEM) than with light microscope (LM). The surface ornamentation of spores is of different orders of magnitude, and fine ornamentation is sometimes revealed by SEM, for example, that of the basidiospores of *Hymenogaster luteus* (Hawker and Madelin 1975). It was found that the spores of the three *Fomes* species did not exhibit any particular surface ornamentation such as ridges, spines, warts, reticulations, or pits. All the spores were smooth.

Although the spores of many higher fungi have clearly defined germ pores, and they are commonly present in the basidiospores of many agarics (Pegler and Young 1971), no germ pores were observed in the *Fomes* species either by LM or SEM observation.

The abscission structure of the higher Basidiomycetes - the apiculus - is difficult to determine by LM. The SEM has elucidated the apiculus structure of the *Fomes* spore, which is situated eccentrically





at the base of the spore. Pegler and Young (1971) recognized two types of apiculus among species of the Agaricales: the nodulose type characteristic of many species with hyaline spores, consisting of a circular area bearing small protuberances but with no indication of a pore; and the open type, consisting of a small depression usually with a pore at the center, found only in species with pigmented spores. The spores of the three *Fomes* species were all hyaline and appear to be similar to the nodulose type, although *Fomes* is classified in the Aphyllophorales.

During spore germination an initial swelling phase appears to be typical of most fungus spores, although many exceptions have been noted. Spore swelling has been described for *Polyporus pargamensis* (Rhoads 1918), *P. gilvus* (Hirt 1927), *F. pinicola* (Mounce 1929), and *Lentinus edodes* (Nakai and Ushiyama 1974), all wood-decay fungi. In this study, it was found that *F. cajanderi* spores became swollen during germination, while spores of *F. fomentarius* and *F. igniarius* did not. It can be said that the tendency of spores of a certain species to swell or not during germination depends, at least in part, on the plasticity or elasticity of the cell wall. In the case of *F. cajanderi* the spore cell wall is quite elastic, since the spores swelled from approximately  $7.5 \times 2.8 \mu\text{m}$  to  $11.1 \times 8.4 \mu\text{m}$ . On the other hand, *F. fomentarius* and *F. igniarius* spores did not exhibit a noticeable water imbibition, indicating that the cell wall elasticity is very low. Different *Fomes* species may have different degrees of cell wall elasticity, which determines the amount of spore swelling.

The germ tubes of the majority of fungus spores may emerge through any part of the spore wall, but in some they emerge through the apex or



at other definite positions. Among wood-decay fungi there appear to be different modes in germ-tube emergence especially as to the number of tubes and presence or absence of polarity. Hirt (1927) reported single germ-tube emergence for *P. gilvus*, while Mounce (1929) reported that the swollen spores of *F. pinicola* produced one to four germ-tubes. Emergence of two germ tubes from a single spore has also been reported for *P. pargamensis* (Rhoads 1918) and *L. edodes* (Nakai and Ushiyama 1974). In this study, spores of both *F. fomentarius* and *F. cajanderi* produced one to two germ tubes. Germ tube emergence showed definite polarity in *F. fomentarius* spores. Similar observations have been made by Rhoads (1918) for *P. pargamensis*, White (1919) for *F. applanatus*, and Nakai and Ushiyama (1974) for *L. edodes*. On the other hand, definite polarity in germ tube emergence was not observed with *F. cajanderi*, and the swollen spores appeared to produce germ tubes at random. Similarly, Mounce (1929) reported that the spores of *F. pinicola* produced their germ tubes from the ends or sides of the oval spores. Thus, it seems that the mode of spore germination varies with different species.

With regard to the preparation for SEM, two methods were used in this study; freeze-drying and critical-point drying. The former method seems to give more reliable surface feature of the spores than the latter, since it exhibited the presence of a mucous-like substance on the spore surface. However, this method appears to create some shrinking effects on the spore walls. On the other hand, although the latter method may be inferior in preserving the mucous-like substance



no shrinking effect was observed and a more precise shape of the spores was preserved. In order to offset the disadvantages of both methods, it is recommended that the two methods be used in the observation of germinating spores. It should be noted also that the purity of the chemicals used for SEM preparation is very critical. As seen in Plate 15-B, a tremendous amount of precipitation was observed on the surface of materials, when crude ethanol was used for the dehydration procedure.

In recent years the ultrastructural events associated with spore germination in fungi have been extensively studied. The study of the internal structure of dormant spore is complicated by the structure and nature of the spore wall, since the cell wall usually consists of more than one layer. In addition, on the layers, particularly in resting spores, may be largely impermeable to the fixatives and stains used and may provide a mechanical obstacle to satisfactory preparation of ultrathin sections (Hawker and Madelin 1975). It was found that ungerminated spores generally exhibited less differentiation in the cell organelles than germinated spores, even if the same fixation method was applied. This may be due to the differences in permeability of the cell wall between ungerminated and germinated spores. Great care must be taken to obtain good fixation of the fungal spore wall by using procedure such as a prolonged fixation period.

From the results, it is clear that the cell wall thickness varied among the three *Fomes* species. The cell wall of ungerminated spores of *F. fomentarius* and *F. igniarius* had two layers, and was quite different from that of *F. cajanderi* which showed only one wall layer. A more conspicuous difference between *F. fomentarius* and *F. cajanderi* spores was observed in the cell wall of the germinated spores. The germ tube





wall of *F. fomentarius* was continuous with the newly formed cell wall layer of the spore, while that of *F. cajanderi* was continuous with the original spore cell wall.

Bartnicki-Garcia (1968) suggested three basically different mechanisms of vegetative wall formation during spore germination. Each type is characteristic of certain groups of fungi. The first type is *de novo* formation of a cell wall on a naked protoplast, and is observed in the encystment of zoospores. The second type is *de novo* formation of a vegetative wall under the spore wall, as is the case in *Rhizopus* sporangiospores. The third type of germination is the extension of the spore wall, or one of its innermost layers. This type is found commonly in the Ascomycetes, Basidiomycetes and Deutromycetes (Hawker and Hendy 1963; Tanaka and Yanagita 1963; Akai and Ishida 1968; Hawker 1966; Ishizaki et al. 1974; Hashimoto et al. 1958; Conti and Naylor 1960; Lowry and Sussman 1968; Manocha and Shaw 1967). For basidiospores, only the third type has been reported. Spores of *Lenzites saepiararia* are known to possess a notably thin spore wall and to show an extension of the spore wall during germination (Hyde and Walkinshaw 1966). Spores of *Psilocybe* species (Stocks and Hess 1970) and *Coprinus lagopus* (Heintz and Niederpruem 1971) were reported to exhibit several layers in their spore wall and the germ tubes were formed from the innermost layer of the spore cell wall of both species.

The results of the present study show that spore germination of *F. cajanderi* is of the third type, since its spore cell wall is very thin and extends to form the germ tube. The type of *F. cajanderi* spore wall is more like that of *L. saepiararia* than that of *Psilocybe* species and *C. lagopus*. However, the cell wall of *F. fomentarius* exhibited striking



changes during germination, which indicates that the type of spore germination is the second type suggested by Bartnicki-Garcia (1968). In this species two new layers were formed inside the original spore wall and the germ tubes were enveloped by a newly-formed elastic, inner wall. There is no published report of this type of spore germination for Basidiomycetes. Thus, it appears that cell wall layer of ungerminated spores varies in different fungal species and the type of germ tube wall formation differs from species to species. Although it is too early to draw conclusions, since too little is known on this aspect, these observations suggest the strong possibility that features of the spore wall and germ tube development may be useful as taxonomic characters. During spore germination *F. fomentarius* and *F. cajanderi* often showed septum formation in the original spores. The reason for the septum formation in the spore is not known.

Cell membranes of *L. saepiaria* and *C. lagopus* basidiospores have been reported to show invagination at irregular intervals by Hyde and Walkinshaw (1966), and Heintz and Niederpruem (1971), respectively. Stocks and Hess (1970) stated that the cell wall membrane of *Psilocybe* species basidiospores normally contained numerous invaginations and in fractured freeze-etched material the invaginations could be seen as randomly arranged channels. In this study the spores of *F. fomentarius* showed increased invagination of the cell membrane when germination took place. In addition, the cell membrane became more noticeable in germinated spores. A similar observation was made by Malhotra and Tewari (1973) who reported that the membrane of the germinating sporangiospores of *Phycomyces* was more conspicuous than that of the dormant spores, and the authors suggested that saturated fatty-acids



in the membrane of the dormant spores might change during germination into unsaturated fatty-acids which react with the fixative more easily.

Basidiospores of *F. fomentarius* and *F. cajanderi* were uninucleate when observed under light microscope and transmission electron microscope. Hawker (1966) mentioned that an early event in spore germination was the increase in nuclear volume, but such a change was not observed in this study. It appeared that nuclear division occurred either before or during germ tube emergence and that one of the daughter nuclei migrated into germ tube. Similar observation was made by Fletcher (1969) with *Penicillium griseofulvum*.

Mitochondrial morphology varies according to the organism, stage of development, and external influences (Bracker 1967). Voelz and Niederpruem (1964) reported that the mitochondria of *Schizophyllum commune* basidiospores were poorly defined and had few cristae. Similarly, in this study the mitochondria of the ungerminated spores of *F. fomentarius* and *F. cajanderi* were not clear, but those of the germinated spores became more distinct. An increase in number and changes in the shape of mitochondria have frequently been observed during germination.

Poor development of endoplasmic reticulum (ER) in dormant spores has been reported in a number of investigations for a variety of spore types (Hawker and Abbott 1963; Voelz and Niederpruem 1964; Stocks and Hess 1970), while prominent ER has been observed in others (Buckley *et al.* 1968; Florance *et al.* 1972; Steele and Fraser 1973). In this study cisternae of ER were more prominent in the ungerminated spores of *F. fomentarius* and *F. cajanderi* than in the germinated spores. There was no marked increase in ER with germination, which is the general





opinion (Robb 1972).

As storage products, lipid bodies and patches of glycogen granules are frequent in dormant spores. In the ungerminated spores of *F. fomentarius* and *F. igniarius* numerous lipid bodies were found throughout the cytoplasm and these tended to disappear or decrease upon germination. This has been also observed during spore germination of *Mucor rouxii* (Bartnicki-Garcia et al. 1968), *Cunninghamella elegans* (Hawker et al. 1970) and *Aspergillus fumigatus* (Campbell 1971). On the other hand, in the ungerminated and germinated spores of *F. cajanderi*, fewer lipid bodies were observed. Voetz and Niederpruem (1964) suggested, from the results of a histochemical experiment and EM study, that the dormant spores of *S. commune* possessed carbohydrate rather than lipids and in the germinating process the lipid droplets appeared. None of the spores of the three *Fomes* species contained glycogen.

No vacuole was found in the ungerminated spores of these *Fomes* species, while vacuolization was commonly observed in the germinated spores. In general, vacuolization takes place during basidiospore germination (Voeltz and Niederpruem 1964; Hyde and Walkinshaw 1966; Stocks and Hess 1970; Aitken and Niederpruem 1970; Heintz and Niederpruem 1971; Nakai and Ushiyama 1974). In the early stage of spore germination, the vacuoles seemed small, but later, they enlarged by fusion. In addition, numerous electron-dense amorphous inclusions were observed in the vacuoles. Similar observations have been made by Hyde and Walkinshaw 1966; Aitken and Niederpruem 1970; Heintz and Walkinshaw 1970; Nakai and Ushiyama 1974). Several suggestions have been advanced as to the nature of these inclusions. Hyde and Walkinshaw (1966) suggested that these inclusions (osmiophilic bodies) observed in germinated spores



of *L. saepiaria* were possibly formed by coalescence of the smaller lipid bodies. McKeen (1970) noted that the inclusions in hyphae of *Erysiphe graminis hordei* were lipids. Wilson (1973) discussed two possibilities in his review of lysosomal concept: (1) If the osmiophilic bodies contain hydrolytic enzymes, these bodies may be serving as primary lysosomes that deposit their enzymes into the vacuoles; (2) If these bodies do not contain hydrolytic enzymes, their fusion with vacuoles may provide a way of mobilizing the lipid and protein. Recently, an entirely different suggestion was made by Nakai (1976) who reported that since the inclusions of *L. edodes* were visible only when the germinating spore materials were prefixed with glutaraldehyde and they were tolerant to pepsin, amylase or sodium methoxide treatment, such inclusions in vacuoles were considered to be artifacts due to glutaraldehyde.

Numerous ribosomes were observed in the ungerminated as well as germinated spores of the *Fomes* species. They were reported to be common in basidiospores of *L. saepiaria* (Hyde and Walkinshaw 1966) and *Psilocybe* species (Stocks and Hess 1970). Golgi has been reported only by Nakai and Ushiyama (1974) in *L. edodes* basidiospores, but it was not found in this study, in any one of the *Fomes* species. Membrane complex were observed in the germinated spores of *F. fomentarius* and *F. cajanderi*. These membrane complex seem quite similar to the tubule-membrane complex which was observed in two species of *Sclerotinia* (Tu and Colotelo 1973). Although the authors reported that the tubule-membrane complex was characteristic of paraphyses and not other tissues of apothecia or sclerotia, it appears that the membrane complex is more commonly observed in germinated spores, and conidiophores (Cole and Aldrich 1971).

Only a few studies have been made of cytological changes in



germinating spores by the use of the light microscope (LM) because of the limited resolution and the restricted depth of focus. Most studies have been carried out using the transmission electron microscope (TEM). However, many conventional histochemical techniques are not satisfactory at the TEM level, for the following reasons: (1) the TEM fixatives may modify the staining reactions of cell organelles when they are subsequently stained (Hayat 1974; De Martino, Natali, Bruni and Accinni 1968); (2) many differential stains are not available. In 1968, Feder and O'Brien introduced some new histological methods for plant cells using glutaraldehyde, acrolein, or formalin fixation and glycol-methacrylate embedding medium. With the combination of thick-sections made by ultramicrotome and various staining methods, improved results were obtained. The purpose of this investigation was to apply these techniques, with some modifications, to *F. fomentarius* spores and to describe the cytological changes during spore germination. *F. fomentarius* was selected for this study, since its basidiospores are relatively large (approximately  $12 \times 5 \mu\text{m}$ ). Emphasis was placed on the cell wall and the nature of vacuole inclusions. It was found that stains used did not differentiate each cell wall layer which was observed with TEM. Generally, the cell wall of the Basidiomycetes consists of glucan and chitin (Joppien *et al.* 1972). Since both glucan and chitin can react with Periodic-acid-Schiff (PAS), whether inner and outer layers of cell wall are highly chitinous or relatively free of chitin is not clear. TEM observations showed that the cell wall of the germinated spore consisted of 4-layers, each of which had a different electron density and thickness. This indicates that each of the cell wall layers may possess a different chemical nature. In order to distinguish the chemical nature





of cell wall layers, differential solubilities and histochemical tests on each layer can be carried out, as shown by Graham (1957) with *Tilletia controversa* teliospores which possess a chitin-prominent inner layer and chitin-lacking outermost wall. Although Toluidine Blue (T.B.) reacted with the cell wall, showing dark bluish purple, again, the definite chemical nature of the cell wall cannot be determined, since the chemistry of the reaction between T.B. and cell wall is still not clear.

Vacuoles as well as inclusions were clearly observed with LM. There has been much speculation as to the nature of these inclusions. In the present study, the inclusions reacted with several stains, and exhibited a different staining nature from that of lipid bodies. Therefore, it seems that the chemical nature of the inclusions is not exactly the same as that of lipid bodies. It was found that each staining procedure used showed interesting and characteristic reactions with the fungal spore materials so that cytological changes during germination were clearly observed. Since the chemical basis of T.B. is not well understood, in spite of its valuable polychromatic nature, conclusive data as to the chemical nature of cell wall and vacuole inclusions could not be obtained. Also, PAS reaction on lipid bodies as well as PAS and IKI reaction on vacuole inclusions are not known conclusively. For further investigation, water-soluble glycolmethacrylate or polyvinylpyrrolidone is recommended. More specific staining with enzyme treatment, blockade, or differential solubilization of cell constituents by various solvents will also be required.

In conclusion, the present study indicates that SEM and TEM can be used to study spore morphology and cytology and that they provide additional, incisive information. It is apparent from this study that



distinct and consistent differences in the spore morphology and cytology during germination occur among these *Fomes* species. I consider that comparative studies of spore germination will serve to clarify relationships among these species whose taxonomy is uncertain or confused.



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## APPENDIX 1

### Agar media

#### 2% malt extract agar

Difco Bacto-malt extract	20 g
'Oxoid' Agar (BDH)	10 g
Distilled water	1000 ml

#### 1% water agar

'Oxoid' Agar (BDH)	10 g
Distilled water	1000 ml

#### 2% glucose, 2% malt extract agar

Difco Bacto-Dextrose	20 g
Difco Bacto-malt extract	20 g
Difco Bacto-Agar	20 g
Distilled water	1000 ml



## APPENDIX 2

## Spore variability

- 1) Spores collected simultaneously\* from sporocarps on different trees.

Germinability of spores on 2% malt extract agar after 24-hour incubation

<u>Sporocarp</u>	<u>%</u>
1 - 2	74
2 - 2	44

\* collected on May 16, 1976

- 2) Spores collected simultaneously\* from different sporocarps on the same tree.

Germinability of spores on 2% malt extract agar after 24-hour incubation

<u>Sporocarp</u>	<u>%</u>
2 - 1	76
2 - 5	82

- 3) Spores collected from the same sporocarps on different dates.

Germinability of spores on 2% malt extract agar after 24-hour incubation

<u>Sporocarp</u>	<u>May 16</u>	<u>May 17</u>	<u>July 8</u>
1 - 2	70%	50%	
2 - 2	44%		57%



## APPENDIX 3

## Acridine orange stains

Basic procedures are from Rigler, R., D. Killander, L. Boland and N.R. Ningertz (1969).

A modification made was: Heat denaturation of cell nuclei in 1/15 M  $\text{Na}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer (pH 6.8) containing 4% formaldehyde, instead of 0.015 M Na-citrate (SSC).





## APPENDIX 4

## Ethanol - water series

30% ethanol for 30 minutes

50% ethanol for 30 minutes

70% ethanol for 30 minutes

100% ethanol for 30 minutes



## APPENDIX 5

## Absolute ethanol - amyl acetate series

30% amyl acetate in absolute ethanol for 30 minutes  
50% amyl acetate in absolute ethanol for 30 minutes  
70% amyl acetate in absolute ethanol for 30 minutes  
100% amyl acetate in absolute ethanol for 30 minutes



## APPENDIX 6

## Ethanol series and propylene oxide series

## Ethanol series

50% ethanol for 30 minutes

70% ethanol for 30 minutes

85% ethanol for 30 minutes

90% ethanol for 30 minutes

95% ethanol for 30 minutes

100% ethanol for 30 minutes

100% ethanol for 30 minutes

## Propylene oxide

100% propylene oxide for 30 minutes

100% propylene oxide for 30 minutes

50% propylene oxide for 30 minutes:

50% Araldite mixture for 2-3 days





## APPENDIX 7

## Araldite 502 medium

DDSA (Dodecenyl succinic anhydrite) 49 ml

Araldite 502 49 ml

IMP - 30 (2, 4, 6, Tri-dimethyl aminomethyl phenol) 2 ml



## APPENDIX 8

## Spurrs' standard medium

ERL - 4206 (vinyl cyclohexene dioxide)	10.0 g
D.E.R. 736	6.0 g
Nonenyl succinic anhydride (NSA)	26.0 g
S-1 accelerator (Dimethyl aminoethanol)	0.4 g



## APPENDIX 9

## Epon 812 medium

## Mixture A

Epon 812	62 ml
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DDSA	100 ml
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## Mixture B

Epon 812	100 ml
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NMAI Nadic methyl anhydride	89 ml
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Mix 3 parts of A and 2 parts of B, and add 2% of DMP-30.





## APPENDIX 10

## Stains for histochemistry

## Toluidine Blue (T.B.)

0.5% T.B. in 0.02 M Benzoate buffer (pH 4.4) and 1.0% T.B. in 1% Borax (pH 8.0 - 9.0) were used. After staining with 0.05% and 1.0% T.B. at 60°C for 1 hour and 30 seconds, respectively, the slides were rinsed in running water until the staining intensity of the cells was well differentiated.

## Periodic acid - Schiff (PAS)

For aldehyde blockade, slides were placed in a solution of 5%  $\text{NH}_2\text{OH} \cdot \text{HCl}$  in 8%  $\text{CH}_3\text{COOH} \cdot 3\text{H}_2\text{O}$  (pH 4.7) for 3 hours at room temperature. After rinsing in running water the slides were placed in 1% periodic acid for 30-60 minutes. After rinsing in water, they were placed in Schiff's reagent (Fisher Scientific Company) for 4 hours, followed by rinsing in water.

## IKI

A drop of 0.2% iodine in 2% KI solution was directly applied on slides for 2.5 hours. The slides were rinsed in running water.

## Sudan Black B

Slides were placed in saturated Sudan Black B in 70% ethanol for 2.5 hours, and then differentiated for 2 minutes in 50% ethanol.



## APPENDIX 11

## Analysis of reducing sugar and amino acids in wood and bark

## Preparation of extracts

Twenty grams of each wood and bark of birch, aspen, balsam poplar and spruce was soaked separately in 200 ml of distilled water for 4 days at 4°C, with occasional shaking. The extract was filtered through Whatman No. 1 filter paper, and the wood or bark was rinsed with additional distilled water. The filtrates were collected and made up to 225 ml.

## Analytical methods

Somogyi-Nelson method for reducing sugar (Nelson, 1944) and Rosen method for amino acid (Rosen, 1957) estimation were used.

	Birch		Aspen		Balsam Poplar		Spruce		
	wood	bark	wood	bark	wood	bark	wood	bark	
Sugars	56.0	3.38	19.1	79.3	11.8	78.8	10.1	33.2	μ mole/g of fresh weight
Amino acids	0.4	0	0.73	1.4	1.2	1.5	0	0.28	

















**B30205**